PCT/GB 2003 / 0 0 5 4 1 6
RECEIVED
10 FEB 2004
WIPO PCT

#2

The Patent Office

INVESTOR IN PEOPLE

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed R. Mahoney

Dated 22 January 2004

BEST AVAILABLE COPY

tents Form 1/77

tents 1977
ule 16)

The Patent Office
PATENTS • DESIGNS • COPYRIGHT • TRADE MARKS

THE PATENT OFFICE
M
19 DEC 2002
RULE 97
NEWPORT

1/77

23DEC02 E772663-5 D01559
P01/7700 0.00-0229725.7

The Patent Office

Cardiff Road
Newport
South Wales
NP10 8QQ

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

1. Your reference	JF/LH/WCM-96
2. Patent application number *(The Patent Office will fill in this part)*	0229725.7
3. Full name, address and postcode of the or of each applicant *(underline all surnames)*	University of Wales College of Medicine Heath Park Cardiff CF14 4XN
Patents ADP number *(if you know it)*	7985460001
If the applicant is a corporate body, give the country/state of its incorporation	
4. Title of the invention	Haplotype Partitioning and Growth Hormone SNPs
5. Name of your agent *(if you have one)* "Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	Wynne-Jones Laine & James Morgan Arcade Chambers 33 St Mary Street Cardiff CF10 1AF
Patents ADP number *(if you know it)*	1792002

	Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*
6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number			

	Number of earlier application	Date of filing *(day / month / year)*
7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application		

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:* *a) any applicant named in part 3 is not an inventor, or* *b) there is an inventor who is not named as an applicant, or* *c) any named applicant is a corporate body.* *See note (d))*	Yes

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	57 / in
Claim(s)	–
Abstract	–
Drawing(s)	8 + 8 /

---

10. If you are also filing any of the following, state how many against each item.

Priority documents	–
Translations of priority documents	–
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	–
Request for preliminary examination and search *(Patents Form 9/77)*	–
Request for substantive examination *(Patents Form 10/77)*	–
Any other documents *(please specify)*	–

---

11. I/We request the grant of a patent on the basis of this application.

Signature Wynne-Jones Laine & James

Wynne-Jones Laine & James

Date 19.12.02

---

12. Name and daytime telephone number of person to contact in the United Kingdom

Julie Fyles - 01242 515807

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 08459 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

Human growth hormone 1 (*GH1*) gene expression: complex haplotype-dependent influence of polymorphic variation in the proximal promoter and locus control region

Martin Horan[1], David S. Millar[1], Jürgen Hedderich[2], Geraint Lewis[1], Vicky Newsway[1], Neil Mo[1], Linda Fryklund[3], Annie M. Procter[1], Michael Krawczak[2], David N. Cooper[1]

[1.] Institute of Medical Genetics, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XN, UK.

[2.] Institut für Medizinische Informatik und Statistik, Christian-Albrechts-Universität, Brunswiker Straße 10, 24105 Kiel, Germany.

[3.] Pharmacia AB, Lindhagensgatan 133, SE-11287, Stockholm, Sweden.

Running title: Growth hormone gene promoter haplotypes

Address for correspondence and reprints: Dr David N. Cooper, Institute of Medical Genetics, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XN, UK. Tel: +44 2920 744062 Fax: +44 2920 747603 Email: cooperdn@cardiff.ac.uk

Summary

The proximal promoter region of the human pituitary expressed growth hormone (*GH1*) gene is highly polymorphic, containing at least 15 single nucleotide polymorphisms (SNPs). This variation is manifest in 40 different haplotypes, the high diversity being explicable in terms of gene conversion, recurrent mutation and selection. Functional analysis showed that 12 haplotypes were associated with a significantly reduced level of reporter gene expression whilst 10 haplotypes were associated with a significantly increased level. The former occur more frequently in the general population than the latter. Although individual SNPs contributed to promoter strength in a highly interactive and non-additive fashion, haplotype partitioning identified six SNPs as major determinants of *GH1* gene expression. The prediction and functional testing of hitherto unobserved super-maximal and sub-minimal promoter haplotypes was then used to test the efficacy of the haplotype partitioning approach. Mobility shift assays demonstrated that five SNP sites exhibit allele-specific protein binding. An association was noted between adult height and the mean *in vitro* expression value corresponding to an individual's *GH1* promoter haplotype combination (P=0.028); however, only 2.5% of the variance of adult height was found to be explicable by reference to this parameter. Three additional SNPs, identified within sites I and II of the upstream Locus Control Region (LCR), were ascribed to three distinct haplotypes. A series of LCR-*GH1* proximal promoter constructs were used to demonstrate that (i) the LCR enhanced proximal promoter activity by up to 2.8-fold depending upon proximal promoter haplotype and that (ii) the activity of a given proximal promoter haplotype was also differentially enhanced by different LCR haplotypes. The genetic basis of inter-individual differences in *GH1* gene expression thus appears to be extremely complex.

Introduction

Human stature is a highly complex trait resulting from the interaction of multiple genetic and environmental factors. Since familial short stature is already known to be associated with inherited mutations of the growth hormone (*GH1*) gene (Procter et al. 1998), it appears reasonable to suppose that polymorphic variation in this pituitary-expressed gene can also influence adult height.

The human *GH1* gene is located on chromosome 17q23 within a 66 kb cluster of five related genes (Chen et al. 1989) including the placentally expressed growth hormone gene (*GH2*), two chorionic somatomammotropin genes (*CSH1* and *CSH2*) and a pseudogene (*CSHP1*). The proximal region of the *GH1* gene promoter exhibits a high level of sequence variation with 16 single nucleotide polymorphisms (SNPs) reported within a 535 base-pair stretch (Giordano et al. 1997; Wagner et al. 1997). The majority of these SNPs occur at the same positions in which the *GH1* gene differs from the paralogous *GH2, CSH1*, *CSH2* and *CSHP1* genes, suggesting that they may have arisen through gene conversion (Giordano et al. 1997; Krawczak et al. 1999).

The expression of the human *GH1* gene is also influenced by a Locus Control Region (LCR) located between 14.5 kb and 32 kb upstream of the *GH1* gene (Jones et al. 1995). The LCR contains multiple DNase I hypersensitive sites and is required for the activation of the genes of the GH gene cluster in both pituitary and placenta (Su et al. 2000; Ho et al. 2002). Two DNase I hypersensitive sites (I and II) contain binding sites for the pituitary-specific transcription factor Pit-1 and are responsible for the high level-, somatotrope-specific expression of the *GH1* gene (Shewchuk et al. 1999). In an attempt to identify common genetic factors that might play a role in determining human stature, we have used *in vitro* reporter gene and mobility shift assays to assess the

relative importance of polymorphic variation in both the proximal promoter region and the LCR on *GH1* gene expression.

Materials and Methods

Human subjects

DNA samples were obtained from lymphocytes taken from 154 male British army recruits of Caucasian origin who were unselected for height. Height data were available for 124 of these individuals (mean, 1.76 ± 0.07 m) and the height distribution was found to be normal (Shapiro-Wilk statistic W=0.984, p=0.16). Ethical approval for these studies was obtained from the local Multi-Regional Ethics Committee.

Polymerase chain reaction (PCR) amplification

PCR amplification of a 3.2 kb *GH1* gene-specific fragment was performed using oligonucleotide primers GH1F (5' GGGAGCCCCAGCAATGC 3'; -615 to –599) and GH1R (5' TGTAGGAAGTCTGGGGTGC 3'; 2598 to 2616) [numbering relative to the transcriptional initation site at +1 (GenBank Accession No. J03071)]. A 1.9kb fragment containing sites I and II of the *GH1* LCR was PCR amplified with LCR5A (5' CCAAGTACCTCAGATGCAAGG 3'; -315 to -334) and LCR3.0 (5' CCTTAGATCTTGGCCTAGGCC 3'; 1589 to 1698) [LCR sequence was obtained from GenBank (Accession No. AC005803) whilst LCR numbering follows that of Jin et al. 1999; GenBank (Accession No. AF010280)]. Conditions for both reactions were identical; briefly, 200ng lymphocyte DNA was amplified using the Expand™ high fidelity system (Roche) using a hot start of 98°C 2 min, followed by 95°C 3 min, 30 cycles 95°C 30 s, 64°C 30 s, 68°C 1 min. For the last 20 cycles, the elongation step at 68°C was increased by 5 s per cycle. This was followed by further incubation at 68°C for 7 min.

Cloning and sequencing

Initially, PCR products were sequenced directly without cloning. The proximal promoter region of the *GH1* gene was sequenced from the 3.2 kb *GH1*-specific PCR fragment using primer GH1S1 (5' GTGGTCAGTGTTGGAACTGC 3': -556 to -537). The 1.9 kb *GH1* LCR fragment was sequenced using primers LCR5.0 (5' CCTGTCACCTGAGGATGGG 3'; 993 to 1011), LCR3.1 (5' TGTGTTGCCTGGACCCTG 3'; 1093 to 1110), LCR3.2 (5' CAGGAGGCCTCACAAGCC 3'; 628 to 645) and LCR3.3 (5' ATGCATCAGGGCAATCGC 3'; 211 to 228). Sequencing was performed using BigDye v2.0 (Applied Biosystems) and an ABI Prism 377 or 3100 DNA sequencer. In the case of heterozygotes for promoter region or LCR variants, the appropriate fragment was cloned into pGEM-T (Promega) prior to sequencing.

Construction of luciferase reporter gene expression vectors

Individual examples of 40 different *GH1* proximal promoter haplotypes (Table 1) were PCR amplified as 582 bp fragments with primers GHPROM5 (5' **AGATCT**GACCCAGGAGTCCTCAGC 3'; -520 to -501) and either GHPROM3A (5' **AAGCTT**GCAGCTAGGTGAGCTGTC 3'; 44 to 62) or GHPROM3C (5' **AAGCTT**GCCGCTAGGTGAGCTGTC 3'; 44 to 62) depending on the base at position +59 of the haplotype. To facilitate cloning, all primers had partial or complete non-templated restriction endonuclease recognition sequences added to their 5' ends (denoted in bold above); *Bgl*II (GHPROM5) and *Hind*III (GHPROM3A and GHPROM3C). PCR fragments were then cloned into pGEM-T. Plasmid DNA was initially digested with *Hind*III (New England Biolabs) and the 5' overhang removed with mung bean nuclease (New England Biolabs). The promoter fragment was released by digestion with *Bgl*II (New England Biolabs) and gel purified. The luciferase reporter vector pGL3 Basic was prepared by *Nco*I (New England Biolabs) digestion and the 5' overhang removed with mung bean nuclease. The vector was then digested with *Bgl*II (New England Biolabs) and gel purified. The restricted promoter fragments were cloned into luciferase reporter gene vector

GL3 Basic. Plasmid DNAs (pGL3GH series) were isolated (Qiagen midiprep system) and sequenced using primers RV3 (5' CTAGCAAAATAGGCTGTCCC 3'; 4760 to 4779), GH1SEQ1 (5' CCACTCAGGGTCCTGTG 3'; 27 to 43), LUCSEQ1 (5' CTGGATCTACTGGTCTGC 3'; 683 to 700) and LUCSEQ2 (5' GACGAACACTTCTTCATCG 3'; 1372 to 1390) to ensure that both the *GH1* promoter and luciferase gene sequences were correct. A truncated *GH1* proximal promoter construct (-288 to +62) was also made by restriction of pGL3GH1 (haplotype 1) with *Nco*I and *Bgl*II followed by blunt-ending/religation to remove SNP sites 1-5.

Artificial proximal promoter haplotype reporter gene constructs were made by site-directed mutagenesis (SDM) [Site-Directed Mutagenesis Kit (Stratagene)] to generate the predicted super-maximal haplotype (AGGGGTTAT-ATGGAG) and sub-minimal haplotypes (AG-TTGTGGGACCACT and AG-TTTTGGGGCCACT).

To make the LCR-proximal promoter fusion constructs, the 1.9 kb LCR fragment was restricted with *Bgl*II and the resulting 1.6 kb fragment cloned into the *Bgl*II site directly upstream of the 582 bp promoter fragment in pGL3. The three different LCR haplotypes were cloned in pGL3 Basic, 5' to one of three *GH1* proximal promoter constructs containing respectively a "high expressing promoter haplotype" (H27), a "low expressing promoter haplotype" (H23) and a "normal expressing promoter haplotype" (H1) to yield a total of nine different LCR-*GH1* proximal promoter constructs (pGL3GHLCR). Plasmid DNAs were then isolated (Qiagen midiprep) and sequence checked using appropriate primers.

Luciferase reporter gene assays

In the absence of a human pituitary cell line expressing growth hormone, rat GC pituitary cells (Bancroft 1973; Bodner and Karin 1989) were selected for *in vitro* expression experiments. Rat GC cells were grown in DMEM containing 15% horse serum and 2.5% fetal calf serum. Human HeLa cells were grown in DMEM containing 5% fetal calf serum. Both cell lines were grown at

37°C in 5% CO_2. Liposome-mediated transfection of GC cells and HeLa cells was performed using Tfx™-20 (Promega) in a 96-well plate format. Confluent cells were removed from culture flasks, diluted with fresh medium and plated out into 96-well plates so as to be ~80% confluent by the following day.

The transfection mixture contained serum-free medium, 250ng pGL3GH or pGL3GHLCR construct, 2ng pRL-CMV, and 0.5μl Tfx™-20 Reagent (Promega) in a total volume of 90μl per well. After 1 hr, 200μl complete medium was added to each well. Following transfection, the cells were incubated for 24 hrs at 37°C in 5% CO_2 before being lysed for the reporter assay.

Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega). Assays were performed on a microplate luminometer (Applied Biosystems) and then normalized with respect to Renilla activity. Each construct was analysed on three independent plates with six replicates per plate (i.e. a total of 18 independent measurements). For the proximal promoter assays, each plate included negative (promoterless pGL3 Basic) and positive (SV40 promoter-containing pGL3) controls. For the LCR analysis, constructs containing the proximal promoter but lacking the LCR were used as negative controls.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed on double stranded oligonucleotides that together covered all 16 SNP sites (Table 2). Nuclear extracts from GC and HeLa cells were prepared as described by Berg et al. (1994). Oligonucleotides were radiolabelled with [γ-^{33}P]-dATP and detected by autoradiography after gel electrophoresis. EMSA reactions contained a final concentration of 20mM Hepes pH7.9, 4% glycerol, 1mM $MgCl_2$, 0.5mM DTT, 50mM KCl, 1.2μg HeLa cell or GC cell nuclear extract, 0.4μg poly[dI-dC].poly[dI-dC], 0.4pM radiolabelled oligonucleotide, 40pM unlabelled competitor oligonucleotide (100-fold excess) where appropriate, in a final

volume of 10μl. EMSA reactions were incubated on ice for 60 mins and electrophoresed on 4% PAGE gels at 100V for 45 mins prior to autoradiography. For each reaction, a double stranded unlabelled test oligonucleotide was used as a specific competitor whilst an oligonucleotide derived from the *NF1* gene promoter (5' CCCCGGCCGTGGAAAGGATCCCAC 3') was used as a non-specific competitor. Double stranded oligonucleotides corresponding to the human prolactin (*PRL*) gene Pit-1 binding site (5' TCATTATATTCATGAAGAT 3') and the Pit-1 consensus binding site (5' TGTCTTCCTGAATATGAATAAGAAATA 3') were used as specific competitors for protein binding to the SNP 8 site.

Primer extension assays

Primer extension assays were performed to confirm that constructs bearing different SNP haplotypes utilized identical transcriptional initiation sites. Primer extension followed the method of Triezenberg et al. (1992).

Data normalization

Expression measurements for negative controls (promoterless pGL3 Basic) exhibited considerable variation between plates (Figure 1a). To correct the data for base-line expression and plate effects, the mean activity of the negative controls on a given plate was subtracted from all other activity values on the same plate. The mean (plate-corrected) activity for proximal promoter haplotype 1 (H1) on each plate was then calculated, and all other haplotype-associated activities on the same plate were divided by this value. These two transformations ensured that the mean negative control activity equalled zero whilst the mean activity of H1 equalled unity, independent of plate number. Resulting activity values may thus be interpreted as fold changes in comparison to H1, corrected for both baseline and plate effects. Since no significant plate effect was detectable after transformation, the data were combined over plates. The results of

this normalization procedure are illustrated for H1 in Figure 1b. A procedure similar to that used for the analysis of the proximal promoter haplotypes was also followed for the LCR-promoter fusion construct expression data, using haplotype A as the reference haplotype.

Statistical analysis

Normalized expression levels of the proximal promoter haplotypes were tested for goodness-of-fit to a Gaussian distribution using the Shapiro-Wilk statistic (W) as implemented in procedure UNIVARIATE of the SAS statistical analysis software (SAS Institute Inc., Cary NC, USA). Significance assessment was adjusted for multiple (i.e. 40-fold) testing by setting $p_{critical}=0.05/40\approx0.001$. Using this criterion, the expression levels of two promoter haplotypes were found to differ significantly from a Gaussian distribution viz. H21 ($W=0.727$, $p=0.0002$) and H40 ($W=0.758$, $p=0.0004$). For the other 38 haplotypes, expression levels were regarded as consistent with normality and were therefore subjected to pair-wise comparison using Tukey's studentized range test (SAS procedure GLM). Pair-wise comparison of expression levels between groups of different haplotypes was performed using normal approximation z of the Wilcoxon rank sum statistic (SAS procedure NPAR1WAY).

The SNPs analysed in this study exerted their influence upon proximal promoter expression in a complex and highly interactive fashion. Further, owing to linkage disequilibrium, expression levels associated with individual polymorphisms were found to be strongly interdependent. It was thus expected that a substantial proportion of the observed variation in expression level would be attributable to variation at a small subset of polymorphic sites. In order to assess formally the correlation structure between the SNPs, and to be able to identify an appropriate subset of critical polymorphisms for further study, the residual deviance upon haplotype partitioning was calculated for all possible subsets of proximal promoter SNPs.

For a given partitioning $\{1\ldots m\}=\Pi=\pi_1\cup\ldots\cup\pi_k$ of a set of data points $x_1,\ldots,x_m$, and with $\pi(i)=j$ if $i\in\pi_j$, the residual deviance δ of Π is defined as

$$\delta = \delta(\Pi) = \sum_{i=1}^{m}(x_i - \bar{x}_{\pi(i)})^2 .$$

When the data set was not partitioned at all, then $\delta=\delta(\Pi_0)=421.7$, and the relative residual deviance of any other partitioning Π was defined as $\delta_R(\Pi)=\delta(\Pi)/\delta(\Pi_0)$.

Six SNPs (nos. 1, 6, 7, 9, 11 and 14; see below) were identified as being responsible for a sizeable proportion (~60%) of the residual deviance in expression level at the same time as invoking relatively little haplotype variation. The statistical interdependence of these SNPs was further analysed by means of a regression tree, constructed by recursive binary partitioning using statistics software R (Ihaka and Gentleman 1996). In the tree construction process, the SNPs were used individually as predictor variables at each node so as to select the two most homogeneous subgroups of haplotypes with respect to the response variable (i.e. normalized proximal promoter expression). The node and SNP that served to introduce a new split were chosen so as to minimize δ_R for the partitioning as defined by the terminating nodes ('leaves') of the resulting intermediate tree. This process was continued until all leaves corresponded to individual haplotypes ('fully grown tree'). The reliability of the δ_R estimates was assessed in each step by 10-fold cross-validation and the standard error (SE) was calculated.

Regression analysis of height and proximal promoter expression level *in vitro* was performed for the 124 height-known individuals studied using the CANCORR procedure of the SAS software package. Let $\mu_{nor,h1}$ and $\mu_{nor,h2}$ denote the mean normalized expression levels of the two haplotypes carried by a given individual. The height of individuals not homozygous for H1 (n=109) was modelled as

$$height = a_0 + a_1 \cdot \frac{\mu_{nor,h1} + \mu_{nor,h2}}{2} + a_2 \cdot \frac{\mu^2_{nor,h1} + \mu^2_{nor,h2}}{2} + a_3 \cdot \mu_{nor,h1} \cdot \mu_{nor,h2}$$

and the coefficient of determination, r^2, calculated.

A reduced median network (Bandelt et al. 1995) was constructed for the seven promoter haplotypes (H1 – H7) that were observed at least 8 times in the 154 study individuals.

Linkage disequilibrium analysis

Linkage disequilibrium (LD) between promoter SNPs, and between SNPs and LCR haplotypes, was evaluated in 100 individuals randomly chosen from the total of 154 under study, using parameter ρ as devised for biallelic loci by Morton et al. (2001). Whilst ρ=1 is equivalent to two loci showing complete LD, ρ=0 indicates complete lack of LD. Only eight SNPs were found to be sufficiently polymorphic in the population sample (heterozygosity ≥5%) to warrant inclusion. SNP5 was excluded owing to its perfect LD with SNP4 (only two pair-wise haplotypes present). Maximum likelihood estimates of the combined LCR-proximal promoter haplotype frequencies, as required for LD analysis, were obtained using an in-house implementation of the expectation maximization (EM) algorithm.

Results

Proximal promoter polymorphism frequencies and haplotypes

The *GH1* gene promoter region has been reported to contain 16 polymorphic nucleotides within a 535 bp stretch (Table 3; Giordano et al. 1997; Wagner et al. 1997). These SNPs were enumerated 1-16 for ease of identification (Figure 2). In a study of 154 male British Caucasians, 15 of these SNPs (all except no. 2) were found to be polymorphic (minor allele frequencies 0.003 to 0.41; Table 3). Variation at the 16 positions was ascribed to a total of 36 different promoter haplotypes (Table 1). Haplotype 1 (H1) may thus be described by a sequence of 16 bases (GGGGGGTATGAAGAAT), representing the 16 SNP locations from –476 to +59. The frequency of the 36 promoter haplotypes varied from 0.339 for H1, henceforth referred to as 'wild-type', to 0.0033 (nos. 25-36) (Table 1). A further 4 haplotypes (nos. 37-40) were found as part of a separate study in 4 individuals exhibiting short stature (Table 1). These haplotypes were absent from the study group but were included in the subsequent analysis for the sake of completeness.

Proximal promoter haplotypes and relative promoter strength

The 40 promoter haplotypes were studied by *in vitro* reporter gene assay and found to differ with respect to their ability to drive luciferase gene expression in rat pituitary cells (Table 4). Expression levels were found to vary over a 12-fold range with the lowest expressing haplotype (no. 17) exhibiting an average level that was 30% that of wild-type and the highest expressing haplotype (no. 27) exhibiting an average level that was 389% that of wild-type (Table 4). Twelve haplotypes (nos. 3, 4, 5, 7, 11, 13, 17, 19, 23, 24, 26 and 29) were associated with a significantly reduced level of luciferase reporter gene expression by comparison with H1. Conversely, a total of 10 haplotypes (nos. 14, 20, 27, 30, 34, 36, 37, 38, 39 and 40) were associated with a significantly increased level of luciferase reporter gene

expression by comparison with H1 (Table 4). Constructs bearing different SNP haplotypes were shown by primer extension assay to utilize identical transcriptional initiation sites (data not shown). Expression of the reporter gene constructs was found to be 1000-fold lower in HeLa cells than in GC cells (data not shown).

The *in vitro* expression levels of the 40 different *GH1* promoter haplotypes are presented graphically in Figure 3. A tendency is apparent for the low expressing haplotypes to occur more frequently whereas the high expressing haplotypes tend to occur less frequently (Wilcoxon $P<0.01$). Since this finding is suggestive of the action of selection, selection effects were sought at the level of individual SNPs. For the 15 SNPs studied here, the mean expression level (weighted by haplotype frequency) and the frequency of the rarer allele in controls were found to be positively correlated (Spearman rank correlation coefficient, $r = 0.32$). If SNP 7 is excluded as an outlier (it has a particularly high expression level associated with the rarer allele), $r = 0.53$ with a one sided $p<0.05$.

The *in vitro* expression level associated with the truncated promoter construct lacking SNPs 1-5 was 102±5% that of the wild-type (haplotype 1). Thus it may be inferred that SNPs 1-5 are likely to have a limited direct influence on *GH1* gene expression.

Expression levels associated with individual SNPs were found to be strongly interdependent. An attempt was therefore made to partition the expression data in such a way as to identify a subset of key polymorphic sites that contribute disproportionately to the observed variation in *in vitro* expression level. Partitioning by the full haplotype comprising all 16 SNPs yielded a relative residual deviance of $\delta_R(\Pi_{16})=0.245$. This can be interpreted in terms of 24.5% of the variation in expression level not being accountable by variation in haplotype. For $1\leq k<16$, the minimum-δ_R-partitioning $\Pi_{k,min}$ was defined as that haplotype partitioning with k SNPs that yielded the smallest relative residual deviance δ_R. The relationship between k and $\delta_R(\Pi_{k,min})$, together with the

number of haplotypes comprising $\Pi_{k,min}$, is depicted in Figure 4. A qualitative difference was evident between k=6 and k=7 in that the number of haplotypes associated with $\Pi_{k,min}$ increases from 13 to 22 whilst $\delta_R(\Pi_{k,min})$ decreases only marginally [$\delta_R(\Pi_{6,min})$=0.397 vs $\delta_R(\Pi_{7,min})$=0.371]. It was therefore concluded that SNPs 1, 6, 7, 9, 11 and 14, which define $\Pi_{6,min}$, represented a good choice of key polymorphisms for further analysis. Of the remaining SNPs, six (nos. 3, 4, 8, 10, 12, and 16) could be classified as "marginally informative". These markers, in combination with the six key SNPs, together define 39 of the 40 haplotypes observed, and account for virtually all of the explicable deviance ($\delta_R(\Pi_{12,min})$=0.245). The other four SNPs (nos. 2, 5, 13 and 15) were "uninformative" with respect to the normalized *in vitro* expression level since they were either monomorphic in our sample (no. 2), or were in perfect (nos. 5 and 13) or near perfect (no. 15) linkage disequilibrium with other markers.

The correlation structure of the six key SNPs was next assessed using a series of successively growing (i.e. nested) regression trees. Following convention in regression tree analysis (Therneau and Atkinson 1997), the smallest intermediate tree with a cross-validated δ_R within one SE of that of the fully grown tree was chosen as a representative partitioning (Figure 5). This 'optimal' tree was found to comprise 10 internal and 11 terminal nodes (Figure 6, Table 5). The relative residual deviance of the tree equals δ_R=0.398, thereby accounting for (1-0.397)/(1-0.245) ≈ 80% of the deviance explicable through haplotype partitioning.

The single most important split was by SNP 7 which on its own accounted for 15% of the explicable deviance. The four haplotypes carrying the C allele of this SNP define a homogeneous subgroup (leaf 11) with a mean normalized expression level 1.8 times higher than that of H1. Haplotypes carrying the T allele of SNP 7 were further sub-

divided by SNP 9, with allele T of this polymorphism causing higher expression (μ_{nor}=1.26) than allele G (μ_{nor}=0.84; Wilcoxon z=7.09, p<0.001). The resulting nnTTnn haplotype was split by SNP 6 (G/T), with nGTTnn forming a terminal node (leaf 8) that includes the wild-type haplotype H1. Interestingly, the nTTTnn haplotypes, when sub-divided by SNP 11, manifested a dramatic difference in expression level. Whilst nTTTGn was found to be a low expresser (μ_{nor}=0.64), haplotype nTTTAn exhibited maximum average expression (μ_{nor}=3.89; Wilcoxon z=5.11, p<0.001).

Haplotype nnTGnn for SNPs 7 and 9 was sub-divided by SNPs 14 and 1, with three of the resulting haplotypes forming terminal nodes (leaves 1, 6 and 7). The fourth haplotype, GnTGnA, was an intermediate expresser (μ_{nor}=0.86) that was further split by SNPs 11 and 6. Interestingly, only one particular combination of SNP 14 and 1 alleles resulted in increased expression on the SNP 7 and 9 nnTGnn background (AnTGnG, leaf 7, μ_{nor}=1.83). A similar non-additive effect upon expression was also noted for SNPs 6 and 11 when considered on haplotype GnTGnA: whereas SNP 11 allele A was associated with higher expression than G in combination with SNP 6 allele T (GTTGAA μ_{nor}=1.18 vs GTTGGA μ_{nor}=0.74; Wilcoxon z=7.09, p<0.001), the opposite held true in combination with SNP 6 allele G (GGTGAA μ_{nor}=0.74 vs GGTGGA μ_{nor}=1.04; Wilcoxon z=5.28, p<0.001).

Evolution of haplotype diversity

Of the 15 *GH1* gene promoter SNPs found to be polymorphic in this study, alternative alleles at 14 positions were potentially explicable by gene conversion since they were identical to those in analogous locations in at least one of the four paralogous human genes (Table 3). Comparison with the orthologous growth hormone (GH) gene

promoter sequences of 10 other mammals revealed that the most frequent alleles at nucleotide positions -75, -57, -31, -6, +3, +16 and +25 (corresponding to SNPs 8-15 inclusive) in the human *GH1* gene were strictly conserved during mammalian evolution (Krawczak et al. 1999). Intriguingly, the rarest of the three alternative alleles at the -1 position (SNP 12) in the human *GH1* gene was identical to that strictly conserved in the mammalian orthologues.

A 'Reduced Median Network' (Figure 7) revealed that wild-type haplotype H1 is not directly connected to other frequent haplotypes by single mutational events. The second most common haplotype, H2, is connected to H1 via H23 and H12 whilst the third most common haplotype, H3, is connected to H1 either through a non-observed haplotype or a double mutation. Expansion of this network so as to incorporate further haplotypes was deemed unreliable owing to the small number of observations per haplotype. Furthermore, expansion of the network would have entailed the introduction of multiple single base-pair substitutions. Since these cannot be distinguished from serial rounds of gene conversion between pre-existing haplotypes, the resulting distances in the network would have been unlikely to reflect genuine evolutionary relationships. However, this may safely be assumed to be the case for the network depicted in Figure 7 that connects the seven most frequent haplotypes, since each mutation occurs only once.

A general decline of linkage disequilibrium (LD) with physical distance was noted for most SNPs, with some notable exceptions (Table 6). Thus, SNP 9 was found to be in strong LD with the other SNPs, including SNP 16 which showed comparatively weak LD with all other proximal promoter SNPs. This finding suggests that the origin of SNP 9 was relatively late. However, SNP 10 was found to be in perfect LD with SNP 12 but not SNP 11 (ρ=0.381), whereas SNP 8 was in stronger LD with SNP 11 than with SNP 10 (ρ=0.925 vs 0.687). These anomalous findings suggest that the extant pattern of LD

among the proximal promoter SNPs is unlikely to have arisen solely through recombinational decay with distance, but rather is likely to reflect the action of other mechanisms such as recurrent mutation, gene conversion or selection.

Prediction and functional testing of super-maximal and sub-minimal haplotypes

Based upon the 'optimal' regression tree obtained for the haplotype-dependent proximal promoter expression data, an attempt was made to predict potential "super-maximal" and "sub-minimal" haplotypes in terms of their levels of expression. To this end, alleles of the six key SNPs were chosen taking the mean expression levels of the appropriate leaves of the tree into account (Table 5). Alleles of the remaining SNPs were determined so as to respectively maximize or minimize expression of individual SNPs. Thus, for the predicted super-maximal haplotype, alleles of SNPs 6, 7, 9 and 11 were as in leaf 10 whilst alleles of SNPs 1 and 14 were as in leaf 7. The sub-minimal haplotype was chosen to represent leaf 1 (for SNPs 1, 7, 9 and 14). The best choice of alleles for SNPs 6 and 11 was however somewhat ambiguous since leaves 2 (suggesting alleles T and G) and 4 (suggesting alleles G and A) predicted similarly low mean expression levels. Therefore, it was decided to generate both constructs for *in vitro* testing. Completion of the hypothetical haplotypes for the remaining SNPs yielded

super-maximal haplotype AGGGGTTAT-ATGGAG and

sub-minimal haplotypes AG-TTGTGGGACCACT, AG-TTTTGGGGCCACT.

These three artificial haplotypes were then constructed and expressed in rat pituitary cells yielding respectively expression levels of 145±4, 55±5 and 20±8% in comparison to wild-type (haplotype 1).

Differences between SNP alleles revealed by mobility shift (EMSA) assay

EMSAs were performed at all proximal promoter SNP sites for all allelic variants using rat pituitary cells as a source of nuclear protein. Protein interacting bands were noted at sites -168, -75, -57, -31, -6/-1/+3 and +16/+25 (Table 7). Inter-allelic differences in the number of protein interacting bands were noted for sites -75 (SNP 8), -57 (SNP 9), -31 (SNP 10), -6/-1/+3 (SNPs 11, 12, 13) and +16/+25 (SNPs 14, 15) [Figure 8; Table 7]. In the case of the latter two sites, EMSA assays on specific SNP allele combinations suggested that differential protein binding was attributable to allelic variation at SNP sites 12 and 15 respectively (Table 7). When the analysis was repeated using a HeLa cell extract, only position -57 manifested evidence of a protein interaction and then only for the G allele, not the T allele (data not shown). The results of competition experiments utilizing oligonucleotides corresponding to two distinct Pit-1 binding sites were consistent with one of the two SNP 8 interacting proteins being Pit-1 (Figure 8). However, the allele-specific protein interaction remained unaffected implying that the other protein involved was not Pit-1.

Association between promoter haplotype expression in vitro and stature in vivo

An attempt was made to correlate the haplotype-specific *in vitro* expression of the *GH1* proximal promoter with adult height in 124 male Caucasians. Each haplotype was ascribed its mean expression value from normalized *in vitro* expression data (Table 4) and the average $A_x=(\mu_{nor,h1}+\mu_{nor,h2})/2$ of the two haplotypes was calculated for each individual. Individuals homozygous for H1 were excluded from the analysis since their A_x values (1.0) would not have contributed any causal variation. This yielded a sample of 109 height-known individuals with suitable genotypes (Table 8). When height above and below the median (1.765 m) was compared to A_x values above and below the median (0.9), evidence for an association between height and *GH1* proximal promoter

haplotype-associated *in vitro* expression emerged (χ^2=4.846, 1 d.f., P=0.028). This notwithstanding, regression analysis using a 2nd degree polynomial demonstrated that the two μ_{nor} values were on their own relatively poor predictors of height. Since the coefficient of determination was r^2=0.025, it may be concluded that approximately 2.5% of the variance in body height is accounted for by reference to *GH1* gene proximal promoter haplotype expression *in vitro*.

Locus control region (LCR) polymorphisms and proximal promoter strength

Three novel polymorphic changes were found within sites I and II (required for the pituitary-specific expression of the *GH1* gene; Jin et al. 1999) of the *GH1* LCR in a screen of 100 individuals randomly chosen from the study group. These were located at nucleotide positions 990 (G/A; 0.90/0.10), 1144 (A/C; 0.65/0.35) and 1194 (C/T; 0.65/0.35) [numbering after Jin et al. 1999]. The polymorphisms at 1144 and 1194 were in total linkage disequilibrium, and three different haplotypes were observed: haplotype A (990G, 1144A, 1194C; 0.55), haplotype B (990G, 1144C, 1194T; 0.35) and haplotype C (990A, 1144A, 1194C; 0.10).

In order to determine whether the three LCR haplotypes exert a differential effect on the expression of the downstream *GH1* gene, a number of different LCR-*GH1* proximal promoter constructs were made. The three alternative 1.6 kb LCR-containing fragments were cloned into pGL3, directly upstream of three distinct types of proximal promoter haplotype, viz. a "high expressing promoter" (H27), a "low expressing promoter" (H23) and a "normal expressing promoter" (H1), to yield nine different LCR-*GH1* proximal promoter constructs in all. These constructs were then expressed in both rat GC cells and HeLa cells, and the resulting luciferase activities measured. In GC cells, the presence of the LCR enhances expression up to 2.8-fold as compared to the proximal

promoter alone (Table 9). However, the extent of this inductive effect was dependent upon the linked promoter haplotype. Two-way analysis of variance (Table 10) revealed that both main effects and the promoter*LCR interaction were significant, with the major influence exerted by the proximal promoter. Also included in Table 9 are the results of a Tukey studentized range test at 95% significance level, performed individually for each promoter haplotype. In conjunction with promoter haplotype 1, the activity of LCR haplotype A is significantly different from that of N (construct containing proximal promoter but lacking LCR), but not from that of LCR haplotypes B and C; LCR haplotypes B and C differ significantly from each other and from N. With promoter 27, however, no significant difference was found between LCR haplotypes. No LCR-mediated induction of expression was noted with any of the proximal promoter haplotypes in HeLa cells (data not shown).

Since the physical distance between the LCR and the proximal promoter SNPs was too great to permit joint physical haplotyping, the linkage disequilibrium (LD) between them was assessed by maximum likelihood methods using genotype data from the 100 individuals included in the analysis of inter-SNP LD for the proximal promoter. Pair-wise LD between promoter SNPs and LCR haplotypes was found to be high for all SNPs except SNP 16 (Table 6). It may therefore be concluded that SNP 16 was subject to recurrent mutation prior to the genesis of SNP 9, the only SNP found to be in strong linkage disequilibrium with SNP 16. Substantial differences between LCR haplotypes exist in terms of their LD with SNPs 4, 8 and 16 (Table 6), suggesting a relatively young age for LCR haplotype B as opposed to haplotype A.

Discussion

Evidence that genetic factors play a major role in determining stature comes from a variety of sources: from intra-familial resemblance (Preece 1996) and twin study-based heritability estimates (Chatterjee et al. 1999) to genome-wide linkage analyses (Hirschhorn et al. 2001). One practical consequence of the high degree of heritability is that the prediction of a child's target height must take parental height into consideration (Luo et al. 1998). Familial short stature has already been shown to be associated with inherited mutations of the growth hormone (*GH1*) gene (Procter et al. 1998). Since the degree of polymorphism exhibited by the *GH1* gene proximal promoter region (16 SNPs in 535 bp) is extremely high, some 30-fold higher than the average for genomic DNA (Brookes 1999; Patil et al. 2001), it appeared worthwhile to explore the proposition that polymorphic variation in this promoter might influence adult height.

In our study population, variation occurred at 15 of the 16 SNP locations and manifested itself in a total of 40 different promoter haplotypes. Twelve haplotypes were found to be associated with a significantly reduced level of luciferase reporter gene expression by comparison with haplotype 1, whereas 10 haplotypes were associated with a significantly increased level. The inverse relationship noted between *GH1* haplotype expression level *in vitro* and population prevalence is intriguing. It may be that natural selection has acted so as to increase the frequency of low expressing haplotypes for reasons quite unrelated to stature e.g. resistance to infection (Saito et al. 1996), starvation (Collins 1995) or trauma (Maison et al. 1998; Takala et al. 1999).

The association noted between *in vitro* promoter haplotype expression and adult height is also remarkable, particularly since expression values were derived from an experimental system that employed a heterologous (rat) pituitary cell line and artificial promoter constructs that lacked the LCR. It follows that our estimate of the variance in

adult height attributable to polymorphic variation in the *GH1* gene promoter (2.5%) is likely to be conservative, and should therefore be regarded as a minimum. Indeed, since the influence of polymorphic variation within the coding region, introns and 3' flanking region of the *GH1* gene (Low et al. 1989; Zhang et al. 1992; Kolb et al. 1998) have not been measured in this analysis, the influence of *GH1* gene variation on both *GH1* gene expression and adult height could have been underestimated. Although GH is a major regulator of human post-natal growth, the hypothalamic/pituitary GH axis includes (or is influenced by) many other factors encoded by multiple genes (e.g. *GHR*, *POU1F1*, *SHOX*, *IGF1*, *LHX3*, *GHRH* and *GHRHR*) [reviewed by Pfäffle et al. 2000]. It is thus reasonable to suppose that these genes may also harbour genetic variants that contribute to the variance of human stature.

From the haplotype frequencies observed in our study group, it is predicted that some 8.2% of the normal population possess two low expressing *GH1* proximal promoter haplotypes (either identical or non-identical) that are associated with *in vitro* GH production ≤55% that of the wild-type. It remains to be seen whether such haplotype combinations occur disproportionately in individuals of short stature; their possession could increase the likelihood that such individuals would come to clinical attention.

Various *cis*-acting regulatory sequences have been identified in the proximal promoter region of the human *GH1* gene. These sequences include binding sites for NF1 (-286 to -274; Courtois et al. 1990), Sp1 (-136 to -127; Lemaigre et al. 1989a), the pituitary-specific transcription factor, Pit-1 (-132 to -107, -92 to -67; Lemaigre et al. 1989b), the vitamin D receptor (VDRE; -60 to -46, -37 to -31; Alonso et al. 1998; Seoane et al. 2002) and CREB, a protein that interacts with cAMP-responsive elements (-188 to -184, -100 to -96; Shepard *et al.*, 1994). Some of these factors may exert their

effects synergistically whereas others appear to bind to promoter motifs in a mutually exclusive fashion. Inspection of the *GH1* gene promoter region suggests that some of the 15 SNPs are located within transcription factor binding sites (Figure 2). Thus, three SNPs cluster around the transcriptional initiation site (SNPs 11-13), one occurs at the 3' end of the proximal VDRE adjacent to the TATA box (SNP 10), one within the distal VDRE (SNP 9), one within the proximal Pit-1 binding site (SNP 8) and one within an NF1 binding site (SNP 6). Expression analysis of a truncated promoter construct was consistent with the limited influence of SNPs 1-5 on *GH1* gene expression. Intriguingly, the nucleotide positions corresponding to SNPs 8 to 15 are strictly conserved in other mammals, a finding which while compatible with their candidacy as functional polymorphisms, nevertheless represents a *caveat* for the interpretation of phylogenetic footprinting studies (Krawczak et al. 1999). Partitioning of the haplotypes identified six SNPs (nos. 1, 6, 7, 9, 11 and 14) as major determinants of *GH1* gene expression level, with a further six SNPs being marginally informative (nos. 3, 4, 8, 10, 12 and 16). The functional significance of all 16 SNPs was investigated by EMSA assays which indicated that six polymorphic sites in the *GH1* proximal promoter interact with nucleic acid binding proteins; for 5 of these sites [-75 (SNP 8), -57 (SNP 9), -31 (SNP 10), -1 (SNP 12) and +25 (SNP 15)], alternative alleles exhibited differential protein binding.

Despite the evident non-additivity of the effects of individual SNP alleles on *GH1* gene expression, an attempt was made to predict potential super-maximal and sub-minimal haplotypes in terms of their expression levels. When tested, one of the sub-minimal haplotypes did indeed manifest a lower level of expression than any naturally occurring haplotype, a result which indicates the efficacy of the process of haplotype partitioning. However, with the other two artificial haplotypes tested, success in obtaining predicted levels of expression was only partial. Thus although certain key

SNPs are identifiable as exerting a disproportionate effect on the expression level associated with a particular haplotype, the expression associated with novel SNP combinations is not entirely predictable. It follows that promoter haplotypes should perhaps to be considered in *Gestalt* terms rather than simply as sums of their component parts.

The molecular basis for haplotype-dependent differences in *GH1* gene promoter strength may thus lie in the net effect of the differential binding of multiple transcription factors to alternative versions of their cognate binding sites. The alternative versions of these sites differ by virtue of their containing different alleles of the various SNPs that combinatorially constitute the observed array of promoter haplotypes. The transcriptional activation of human genes is mediated by the interaction of transcription factors with different combinations and permutations of their cognate binding sites on the gene promoter. Some transcription factors are coordinated directly by *cis*-acting DNA sequence motifs, others indirectly by protein–protein interactions in what has been likened to a three-dimensional jigsaw puzzle: the DNA sequence motifs providing the puzzle template, the transcription factors constituting the puzzle pieces. This modular view of the promoter helps one to envisage how the effect of different SNP combinations in a given haplotype might be transduced so as to exert differential effects on transcription factor binding, transcriptosome assembly and hence gene expression. Thus, for example, the observed non-additive effects of *GH1* promoter SNPs on gene expression may be understood in terms of the allele-specific differential binding of a given protein at one SNP site affecting in turn the binding of a second protein at another SNP site that is itself subject to allele-specific protein binding.

This study represents the first direct evidence for the existence of functional polymorphisms in the *GH1* gene. It has previously been claimed that *GH1* SNPs at both

-278 (P-2) and 1169 in intron 4 (P-1) are associated with both height and GH secretion after provocative testing (Hasegawa et al. 2000). However, no evidence for any direct effect of the -278 SNP was presented in this study, and the association with P-1 may have been due to linkage disequilibrium. Although the intronic polymorphism has recently been reported to be associated with colorectal neoplasia (Le Marchand et al. 2002), no association was apparent between P-1 and any of the promoter haplotypes reported here.The LCR upstream of the GH gene cluster contains sequence elements that possess enhancer activity, confer tissue specificity of expression, and promote long range gene activation through the spreading of histone acetylation (Shewchuk et al. 1999; Su et al. 2000; Shewchuk et al. 2001; Ho et al. 2002). The somatotrope-specific determinants of the LCR are present within a 1.6 kb region (sites I and II) ~14.5 kb upstream of the *GH1* gene (Shewchuk et al. 1999). In our own system, the introduction of this 1.6 kb LCR fragment served to enhance the activity of the *GH1* proximal promoter by up to 2.8-fold, although the degree of enhancement was found to be dependent upon the identity of the linked proximal promoter haplotype. Conversely, enhancement of the activity of a proximal promoter of given haplotype was also found to be dependent upon the identity of the LCR haplotype. Taken together, these findings imply that the genetic basis of inter-individual differences in *GH1* gene expression is likely to be extremely complex. In this regard, the results are also reminiscent of the β-globin LCR in which SNPs have been previously reported in the HS2 and HS4 regions (Perichon et al. 1993; Kukreti et al. 2002), with different alleles of the HS2 SNP conferring different levels of enhancement upon the expression of a γ-globin promoter-linked reporter gene (Ofori-Acquah et al. 2001).

Promoter polymorphisms affecting human gene expression are not infrequent and an increasing number have been characterized by functional studies e.g. those in the

plasminogen activator inhibitor type 1 (*PAI1*; Dawson et al. 1993), tumour necrosis factor α (*TNF*; Wilson et al. 1997), apolipoprotein AI (*APOA1*; Angotti et al. 1994) and lipoprotein lipase (*LPL*; Hall et al. 1997) genes. The combinatorial effects of SNPs in promoter regions are evident from studies of disease-associated polymorphism haplotypes. One such example is provided by the *PDGFRA* gene promoter in which the different haplotypes differ in terms of their ability to drive reporter gene expression *in vitro* and are differentially associated with the risk of a neural tube defect (Joosten et al. 2001). Under the assumption of additivity, attempts have sometimes been made to tease out the net effects of individual SNPs by combinatorial functional assay. Our study has however served to demonstrate that SNPs within a promoter haplotype exert their influence on gene expression in a highly complex and interactive fashion. Such non-additive effects are not without precedent, having been reported before in the paraoxonase 1 (*PON1*), interleukin 6 (*IL6*) and β2-adrenergic receptor (*ADRB2*) gene promoter regions (Terry et al. 2000; Brophy et al. 2001; Drysdale et al. 2000) albeit with much smaller numbers of SNPs. If, as appears increasingly likely (Tiret et al. 2002), such complexity were to be a common feature of gene promoters, it would not bode well for the success of conventional DNA polymorphism-disease association studies. Indeed, in cases where non-additivity of individual SNPs pertained, haplotype analysis would offer certain advantages (Bader 2001; Judson and Stephens 2001). The approach described here nevertheless represents a first attempt to partition into its constituent components the effect on gene expression of a complex promoter haplotype whilst concurrently exploring the interactions between those components.

Acknowledgements

We are grateful to Mark Jones and Sarah Maund for technical assistance given during the early stages of this project, and to John Gregory for helpful comments on the manuscript. This work was partially supported by Pharmacia AB, Stockholm, Sweden.

Electronic-Database Information

The Accession numbers and URL for the data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for Accession Nos. AC005803 and AF010280).

References

Alonso M, Segura C, Dieguez C, Perez-Fernandez R (1998) High-affinity binding sites to the vitamin D receptor DNA binding domain in the human growth hormone promoter. Biochem Biophys Res Commun 247:882-887

Angotti E, Mele E, Costanzo F (1994) A polymorphism (G→A transition) in the -78 position of the apolipoprotein A-I promoter increases transcriptional efficiency. J Biol Chem 269:17371-17374

Bader JS (2001) The relative power of SNPs and haplotype as genetic markers for association tests. Pharmacogenomics 2: 11-24.

Bancroft FC (1973) Measurement of growth hormone synthesis by rat pituitary cells in culture. Endocrinology 92:1014-1021

Bandelt H-J, Forster P, Sykes BC, Richards MB (1995) Mitochondrial portraits of human populations. Genetics 141:743-753

Bodner M, Karin M (1987) A pituitary-specific *trans*-acting factor can stimulate transcription from the growth hormone promoter in extracts of nonexpressing cells. Cell 50: 267-275.

Brookes AJ (1999) The essence of SNPs. Gene 234:177-186

Brophy VH, Hastings MD, Clendenning JB, Richter RJ, Jarvik GP, Furlong CE (2001) Polymorphisms in the human paraoxonase (*PON1*) promoter. Pharmacogenetics 11:77-84

Chatterjee S, Das N, Chatterjee P (1999) The estimation of the heritability of anthropometric measurements. Appl Human Sci 18:1-7

Chen EY, Liao Y-C, Smith DH, Barrera-Saldana HA, Gelinas RE, Seeburg PH (1989) The human growth hormone locus: nucleotide sequence, biology and evolution. Genomics 4:479-497

Collins S (1995) The limit of human adaptation to starvation. Nature Medicine 1: 810-814

Courtois SJ, Lafontaine DA, Lemaigre FP, Durviaux SM, Rousseau GG (1990) Nuclear factor-I and activator protein-2 bind in a mutually exclusive way to overlapping promoter sequences and trans-activate the human growth hormone gene. Nucleic Acids Res 18:57-64

Dawson SJ, Wiman B, Hamsten A (1993) The two allele sequences of a common polymorphism in the promoter of the plasminogen activator inhibitor-1 (PAI-1) gene respond differently to interleukin-1 in HepG2 cells. J Biol Chem 268:10739-10745

Drysdale CM, McGraw DW, Stack CB, Stephens JC, Judson RS, Nandabalan K, Arnold K, Ruano G, Liggett SB (2000) Complex promoter and coding region β_2-

adrenergic receptor haplotypes alter receptor expression and predict *in vivo* responsiveness. Proc Natl Acad Sci USA 97:10483-10488

Giordano M, Marchetti C, Chiorboli E, Bona G, Richiardi PM (1997) Evidence for gene conversion in the generation of extensive polymorphism in the promoter of the growth hormone gene. Hum Genet 100:249-255

Hall S, Chu G, Miller G, Cruickshank K, Cooper JA, Humphries SE, Talmud PJ (1997) A common mutation in the lipoprotein lipase gene promoter, -93T/G, is associated with lower plasma triglyceride levels and increased promoter activity *in vitro*. Arterioscl Thromb Vasc Biol 17:1969-1976

Hasegawa Y, Fujii K, Yamada M, Igarashi Y, Tachibana K, Tanaka T, Onigata K, Nishi Y, Kato S, Hasegawa T (2000) Identification of novel human *GH-1* gene polymorphisms that are associated with growth hormone secretion and height. J Clin Endocrinol Metab 85:1290-1295

Hirschhorn JN, Lindgren CM, Daly MJ, Kirby A, Schaffner SF, Burtt NP, Altshuler D, Parker A, Rioux JD, Platko J, Gaudet D, Hudson TJ, Groop LC, Lander ES (2001) Genomewide linkage analysis of stature in multiple populations reveals several regions with evidence of linkage to adult height. Am J Hum Genet 69:106-116

Ho Y, Elefant F, Cooke N, Liebhaber S (2002) A defined locus control region determinant links chromatin domain acetylation with long-range gene activation. Molecular Cell 9:291-302

Ihaka R, Gentleman R (1996) R: A language for data analysis and graphics. J Comput Graph Stat 5:299-314

Jin Y, Surabhi RM, Fresnoza A, Lytras A, Cattini PA (1999) A role for A/T-rich sequences and Pit-1/GHF-1 in a distal enhancer located in the human growth hormone locus control region with preferential pituitary activity in culture and transgenic mice. Mol Endocrinol 13:1249-1266

Jones BK, Monks BR, Liebhaber SA, Cooke NE (1995) The human growth hormone gene is regulated by a multicomponent locus control region. Mol Cell Biol 15:7010-7021

Joosten PHLJ, Toepoel M, Mariman ECM, Van Zoelen EJJ (2001) Promoter haplotype combinations of the platelet-derived growth factor α-receptor gene predispose to human neural tube defects. Nature Genet 27:215-217

Judson R, Stephens JC (2001) Notes from the SNP vs. haplotype front. Pharmacogenomics 2: 7-10.

Kolb AF, Gunzburg WH, Brem G, Erfle V, Salmons B (1998) A functional eukaryotic promoter is contained within the first intron of the hGH-N coding region. Biochem Biophys Res Commun 247:332-337

Krawczak M, Chuzhanova NA, Cooper DN (1999) Evolution of the proximal promoter region of the mammalian growth hormone gene. Gene 237:143-151

Kukreti R, B-Rao C, Das SK, De M, Talukder G, Vaz F, Verma IC, Brahmachari SK (2002) Study of the single nucleotide polymorphism (SNP) at the palindromic sequence of hypersensitive site (HS)4 of the human β-globin locus control region (LCR) in Indian population. Am J Hematol 69:77-79

Le Marchand L, Donlon T, Seifried A, Kaaks R, Rinaldi S, Wilkens LR (2002) Association of a common polymorphism in the human GH1 gene with colorectal neoplasia. J Natl Cancer Inst 94:454-460

Lemaigre FP, Courtois SJ, Lafontaine DA, Rousseau GG (1989a) Evidence that the upstream stimulatory factor and the Sp1 transcription factor bind *in vitro* to the promoter of the human growth hormone gene. Eur J Biochem 181:555-561

Lemaigre FP, Peers B, Lafontaine DA, Mathy-Hartert M, Rousseau GG, Belayew A, Martial JA (1989b) Pituitary-specific factor binding to the human prolactin, growth hormone and placental lactogen genes. DNA 8:149-159

Low MJ, Goodman RH, Ebert KM (1989) Cryptic human growth hormone gene sequences direct gonadotroph-specific expression in transgenic mice. Mol Endocrinol 3:2028-2033

Luo ZC, Albertsson-Wikland K, Karlberg J (1998) Target height as predicted by parental heights in a population-based study. Pediatr Res 44:563-571

Maison P, Balkau B, Simon D, Chanson P, Rosselin G, Eschwège E (1998) Growth hormone as a risk for premature mortality in healthy subjects: data from the Paris prospective study. Br Med J 316:1132-1133

Morton NE, Zhang W, Taillon-Miller P, Ennis S, Kwok OY, Collins A (2001) The optimal measure of allelic association. Proc Natl Acad Sci USA 98:5217-5221

Ofori-Acquah SF, Lalloz MR, Layton DM (2001) Nucleotide variation regulates the level of enhancement by hypersensitive site 2 of the β-globin locus control region. Blood Cells Mol Dis 27:803-811

Patil N, Berno AJ, Hinds DA, Barrett WA, Doshi JM, Hacker CR, Kautzer CR et al.. (2001) Blocks of limited haplotype diversity revealed by high-resolution scanning of human chromosome 21. Science 294:1719-1723

Perichon B, Ragusa A, Lapoumeroulie C, Romand A, Moi P, Ikuta T, Labie D, Elion J, Krishnamoorthy R (1993) Inter-ethnic polymorphism of the β-globin gene locus control region (LCR) in sickle cell anemia patients. Hum Genet 91:464-468

Pfäffle R, Blankenstein O, Wüller S, Heimann K, Heimann G (2000) Idiopathic growth hormone deficiency: a vanishing diagnosis? Horm Res 53 Suppl. 3:1-8

Preece MA (1996) The genetic contribution to stature. Horm Res 45 Suppl. 2:56-58

Procter AM, Phillips JA, Cooper DN (1998) The molecular genetics of growth hormone deficiency. Hum Genet 103:255-272

Saito H, Inoue T, Fukatsu K, Ming-Tsan L, Inaba T, Fukushima R, Muto T (1996) Growth hormone and the immune response to bacterial infection. Horm Res 45:50-54

Seoane S, Alonso M, Segura C, Pérez-Fernández R (2002) Localization of a negative vitamin D response sequence in the human growth hormone gene. Biochem Biophys Res Commun 292:250-255

Shepard AR, Zhang W, Eberhardt NL (1994) Two CGTCA motifs and a GHF1/Pit1 binding site mediate cAMP-dependent protein kinase A regulation of human growth hormone gene expression in rat anterior pituitary GC cells. J Biol Chem 269:1804-1814

Shewchuk BM, Asa SL, Cooke NE, Liebhaber SA (1999) Pit-1 binding sites at the somatotrope-specific DNase I hypersensitive sites I, II of the human growth hormone locus control region are essential for *in vivo hGH-N* gene activation. J Biol Chem 274:35725-35733

Shewchuk BM, Cooke NE, Liebhaber SA (2001) The human growth hormone locus control region mediates long-distance transcriptional activation independent of nuclear matrix attachment regions. Nucleic Acids Res 29:3356-3361

Su Y, Liebhaber SA, Cooke NE (2000) The human growth hormone gene cluster locus control region supports position-independent pituitary- and placenta-specific expression in the transgenic mouse. J Biol Chem 275:7902-7909

Takala J, Ruokonen E, Webster NR, Nielsen MS, Zandstra DF, Vundelinckx G, Hinds CJ (1999) Increased mortality associated with growth hormone treatment in critically ill adults. New Engl J Med 341:785-792

Terry CF, Loukaci V, Green FR (2000) Cooperative influence of genetic polymorphisms on interleukin 6 transcriptional regulation. J Biol Chem 275:18138-18144

Therneau TM, Atkinson EJ (1997) An introduction to recursive partitioning using RPART routines. Technical report #61. The Mayo Foundation, Rochester MN.

Tiret L, Poirier O, Nicaud V, Barbaux S, Herrmann S-M, Perret C, Raoux S, Francomme C, Lebard G, Trégouët D, Cambien F (2002) Heterogeneity of linkage disequilibrium in human genes has implications for association studies of common diseases. Hum Mol Genet 11:419-429

Triezenberg SJ (1992) Primer extension. In Ausubel FM, Brent RE, Kingston DD, Moore JA, Smith JA, Seidman JG, Struhl K (Eds). Current Protocols in Molecular Biology. pp 4.8.1-4.8.5. John Wiley, New York.

Wagner JK, Eblé A, Cogan JD, Prince MA, Phillips JA, Mullis PE (1997) Allelic variations in the human growth hormone-1 gene promoter of growth hormone-deficient patients and normal controls. Eur J Endocrinol 137:474-481

Wilson AG, Symons JA, McDowell TL, McDevitt HO, Duff GW (1997) Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. Proc Natl Acad Sci USA 94:3195-3199

Zhang W, Brooks RL, Silversides DW, West BL, Leidig F, Baxter JD, Eberhardt NL (1992) Negative thyroid hormone control of human growth hormone gene expression is mediated by 3'-untranslated/3'-flanking DNA. J Biol Chem 267:15056-15063

Table 1. *GH1* proximal promoter haplotypes defined by genetic variation at 16 locations

No.	-476	-364	-339	-308	-301	-278	-168	-75	-57	-31	-6	-1	+3	+16	+25	+59	n
	SNP position relative to *GH1* gene transcriptional start site																
1	G	G	G	G	G	G	T	A	T	G	A	A	G	A	A	T	103
2	G	G	G	G	G	T	T	A	G	G	G	A	G	A	A	T	50
3§	G	G	G	T	T	G	T	A	G	G	A	A	G	A	A	T	28
4§	G	G	G	T	T	G	T	A	G	-	A	A	G	A	A	T	16
5§	G	G	G	G	G	T	T	G	G	G	G	A	G	A	A	T	13
6	G	G	G	T	T	G	T	A	G	-	A	A	G	A	A	G	9
7§	G	G	G	G	G	T	T	A	G	G	G	T	G	A	A	T	8
8	G	G	G	T	T	G	T	A	G	G	G	A	G	A	A	T	6
9	G	G	G	G	G	T	T	A	T	G	G	A	G	A	A	T	6
10	G	G	G	T	T	G	T	A	G	-	G	A	G	A	A	T	6
11§	G	G	G	G	G	T	T	G	G	G	G	A	G	G	C	T	5
12	G	G	G	G	G	T	T	A	G	G	A	A	G	A	A	T	5
13§	G	G	-	G	G	T	T	G	G	G	G	A	G	A	A	T	5
14	G	G	G	G	G	T	C	A	G	G	G	T	G	A	A	T	5
15	G	G	G	T	T	G	T	A	G	G	G	T	G	A	A	T	4
16	G	G	G	G	G	T	T	G	G	G	A	A	G	A	A	T	4
17§	G	G	-	G	G	T	T	A	G	G	G	A	G	A	A	T	4
18	G	G	G	G	G	T	T	A	G	-	G	A	G	A	A	T	3
19§	A	G	G	G	G	T	T	A	G	G	G	A	G	A	A	T	3
20	G	G	G	G	G	G	T	A	G	-	A	A	G	A	A	T	3
21	G	G	G	G	G	T	T	G	G	G	G	A	G	A	A	G	3
22	G	G	G	T	T	G	T	A	T	G	A	A	G	A	A	T	3
23§	G	G	G	G	G	G	T	A	G	G	A	A	G	A	A	T	2
24§	G	G	G	T	T	G	T	G	G	-	A	A	G	A	A	T	2
25	G	G	G	T	T	G	T	A	G	G	A	A	G	A	A	G	1
26§	G	G	G	G	G	T	T	G	G	G	G	T	G	A	A	T	1
27	G	G	G	G	G	T	T	A	T	G	A	A	G	A	A	T	1
28	G	G	G	G	G	T	T	A	G	-	A	A	G	A	A	T	1
29§	A	G	G	G	G	T	T	A	G	G	A	A	G	A	A	T	1
30	G	G	-	G	G	T	T	A	G	G	A	A	G	A	A	T	1
31	G	G	G	G	G	T	T	G	G	-	G	A	G	A	A	T	1
32	G	G	G	T	T	G	T	G	G	G	G	A	G	A	A	G	1
33	G	G	G	G	G	T	T	A	G	G	G	A	G	G	C	T	1
34	G	G	-	G	G	T	C	A	G	G	G	T	G	A	A	T	1
35	G	G	G	G	G	G	T	A	G	G	A	C	C	A	A	T	1
36	G	G	G	G	G	T	T	A	G	G	G	T	G	A	A	G	1
37$	A	G	G	G	G	T	T	A	G	G	G	A	G	G	A	T	0
38$	G	G	G	G	G	T	C	A	G	G	A	A	G	A	A	T	0
39$	G	G	G	T	T	G	T	A	G	G	G	A	G	A	C	T	0
40$	G	G	G	G	G	T	C	A	G	G	G	A	G	A	A	T	0

n: frequency in 154 male British Caucasians; §: haplotypes exhibiting a significantly reduced level (55% that of haplotype 1) of luciferase activity in GC cells; $: only found in solitary cases of GH deficiency.

Table 2 Double-stranded oligonucleotide primer sequences for EMSA analysis of SNP sites exhibiting allele-specific protein binding. SNP sites 11 - 15 were studied in different allele combinations. TSS: transcriptional initiation site.

SNP/allele	Position from TSS	Sequence 5'→3'
8 A	-89 → -61	CCATGCATAAATGTACACAGAAACAGGTG CACCTGTTTCTGTGTACATTTATGCATGG
8 G		CCATGCATAAATGTGCACAGAAACAGGTG CACCTGTTTCTGTGCACATTTATGCATGG
9 G	-72 → -42	CAGAAACAGGTGGGGGCAACAGTGGGAGAGA TCTCTCCCACTGTTGCCCCCACCTGTTTCTG
9 T		CAGAAACAGGTGGGGTCAACAGTGGGAGAGA TCTCTCCCACTGTTGACCCCACCTGTTTCTG
10 G	-45 → -15	GAGAAGGGGCCAGGGTATAAAAAGGGCCCAC GTGGGCCCTTTTTATACCCTGGCCCCTTCTC
10 ΔG		GAGAAGGGGCCAGGTATAAAAAGGGCCCAC GTGGGCCCTTTTTATACCTGGCCCCTTCTC
11, 12, 13 A A G	-18 → +15	CCACAAGAGACCAGCTCAAGGATCCCAAGGCCC GGGCCTTGGGATCCTTGAGCTGGTCTCTTGTGG
11, 12, 13 G A G		CCACAAGAGACCGGCTCAAGGATCCCAAGGCCC GGGCCTTGGGATCCTTGAGCCGGTCTCTTGTGG
11, 12, 13 G T G		CCACAAGAGACCGGCTCTAGGATCCCAAGGCCC GGGCCTTGGGATCCTAGAGCCGGTCTCTTGTGG
14, 15 A A	+4 → +37	ATCCCAAGGCCCAACTCCCCGAACCACTCAGGGT ACCCTGAGTGGTTCGGGGAGTTGGGCCTTGGGAT
14, 15 G C		ATCCCAAGGCCCGACTCCCCGCACCACTCAGGGT ACCCTGAGTGGTGCGGGGAGTCGGGCCTTGGGAT
14, 15 G A		ATCCCAAGGCCCGACTCCCCGAACCACTCAGGGT ACCCTGAGTGGTTCGGGGAGTCGGGCCTTGGGAT
14, 15 A C		ATCCCAAGGCCCAACTCCCCGCACCACTCAGGGT ACCCTGAGTGGTGCGGGGAGTTGGGCCTTGGGAT

Table 3: Allele frequencies of 15 SNPs in the *GH1* gene promoter of 154 male Caucasians and corresponding nucleotides in analogous locations of the paralogous genes of the GH cluster

SNP	Position$	*GH1* Allele	Frequency	*GH2*	*GH1* paralogues§ *CSH1*	*CSH2*	*CSHP1*
1	-476	G	304 (0.987)	A	G	G	A
		A	4 (0.013)				
3	-339	G	297 (0.964)	G	G	G	G
		-	11 (0.036)				
4	-308	G	232 (0.753)	T	C	C	T
		T	76 (0.247)				
5	-301	G	232 (0.753)	T	T	T	T
		T	76 (0.247)				
6	-278	G	185 (0.601)	T	A	A	T
		T	123 (0.399)				
7	-168	T	302 (0.981)	T	C	C	T
		C	6 (0.019)				
8	-75	A	273 (0.886)	G	A	A	G
		G	35 (0.114)				
9	-57	G	195 (0.633)	A	T	T	G
		T	113 (0.367)				
10	-31	G	267 (0.867)	-	G	G	G
		-	41 (0.133)				
11	-6	A	181 (0.588)	A	G	G	A
		G	127 (0.412)				
12	-1	A	287 (0.932)	A	T	T	C
		T	20 (0.065)				
		C	1 (0.003)				
13	+3	G	307 (0.997)	G	G	G	C
		C	1 (0.003)				
14	+16	A	302 (0.981)	A	A	A	G
		G	6 (0.019)				
15	+25	A	302 (0.981)	A	A	A	C
		C	6 (0.019)				
16	+59	T	293 (0.951)	G	G	G	G
		G	15 (0.049)				

$: relative to the *GH1* transcription start site; §: bases at the analogous positions in the wild-type sequences of the four paralogous genes in the human GH cluster.

Table 4 *In vitro GH1* gene promoter expression analysis of 40 different SNP haplotypes

Haplotype No.	n	μ_{nor}	σ_{nor}	Tukey
17	18	0.304	0.054	a----------------
3	18	0.324	0.170	a----------------
19	18	0.332	0.062	a----------------
23	18	0.359	0.042	ab---------------
24	18	0.395	0.107	abc--------------
11	18	0.406	0.069	abc--------------
26	18	0.410	0.181	abc--------------
13	18	0.483	0.084	abcd-------------
29	18	0.502	0.149	abcd-------------
4	18	0.528	0.205	abcde------------
5	18	0.536	0.205	abcde------------
7	18	0.553	0.154	abcdef-----------
21	18	0.577	0.206	*
9	18	0.635	0.268	abcdefg----------
15	18	0.725	0.271	abcdefgh---------
25	18	0.790	0.229	-bcdefghi--------
32	18	0.793	0.242	-bcdefghi--------
33	18	0.807	0.225	--cdefghi--------
35	18	0.809	0.230	--cdefghi--------
18	12	0.819	0.217	--cdefghi--------
10	18	0.855	0.135	---defghi--------
12	18	0.958	0.357	----efghij-------
16	18	0.988	0.290	-----fghijk------
1	90	1.000	0.174	------ghijk------
6	18	1.075	0.404	-------hijkl-----
2	18	1.078	0.150	-------hijkl-----
31	18	1.208	0.353	--------ijklm----
28	18	1.317	0.312	---------jklmn---
8	18	1.333	0.453	---------jklmn---
22	18	1.403	0.380	----------klmno--
30	18	1.447	0.345	-----------lmno--
36	18	1.451	0.368	-----------lmno--
39	18	1.468	0.653	-----------lmno--
20	18	1.600	0.342	------------mnop-
38	18	1.697	0.752	-------------nop-
40	18	1.733	1.112	*
14	18	1.806	0.386	--------------op-
37	18	1.825	0.765	--------------op-
34	18	1.997	0.352	---------------p-
27	18	3.890	0.901	----------------q
Negative control	90	0.000	0.005	

n: number of measurements; μ_{nor}: mean normalized expression level (i.e. fold change compared to H1); σ_{nor}: standard deviation of expression level; Tukey: result of Tukey's studentized range test, haplotypes with overlapping sets of letters are not statistically different in terms of their mean expression level; *: non-Gaussian distribution

Table 5 Haplotype partitioning of *GH1* gene promoter expression data

Haplotype§	leaf&	n_{hap}	n	μ_{nor}	σ_{nor}	δ(leaf)
nnCnnn	11	4	72	1.809	0.725	36.27
nGTTnn	8	2	108	1.067	0.267	7.62
nTTTGn	9	1	18	0.635	0.268	1.22
nTTTAn	10	1	18	3.890	0.902	13.82
AnTGnA	1	2	36	0.418	0.142	0.71
GnTGnG	6	2	36	0.607	0.262	2.39
AnTGnG	7	1	18	1.825	0.765	9.95
GTTGGA	2	10	174	0.740	0.427	31.54
GGTGAA	4	8	144	0.735	0.474	32.16
GGTGGA	3	5	90	1.035	0.493	21.66
GTTGAA	5	4	72	1.178	0.384	10.47

n_{hap}: number of haplotypes included in leaf; μ_{nor}: mean normalized expression level; σ_{nor}: standard deviation of expression level; δ(leaf): residual deviance within leaf; §: alleles are given in the order of SNP 1, 6, 7, 9, 11 and 14 (n: any base); &: numbering as in Figure 6.

Table 6 Linkage disequilibrium, ρ, between *GH1* proximal promoter SNPs and LCR haplotypes in 100 male Caucasians

	SNP							
SNP	4	6	8	9	10	11	12[&]	16
4	-.-	1.000	0.802	0.893	0.731	0.554	0.638	0.567
6	1.000	-.-	0.927	0.868	0.632	0.891	0.867	0.111
8	0.802	0.927	-.-	1.000	0.687	0.925	0.242	0.251
9	0.893	0.868	1.000	-.-	1.000	0.905	1.000	1.000
10	0.731	0.632	0.687	1.000	-.-	0.381	1.000	0.415
11	0.554	0.891	0.925	0.905	0.381	-.-	1.000	0.044
12[&]	0.638	0.867	0.242	1.000	1.000	1.000	-.-	0.025
16	0.567	0.111	0.251	1.000	0.415	0.044	0.025	-.-
LCR[$]	4	6	8	9	10	11	12	16
A	0.153	0.829	1.000	0.931	0.601	0.782	0.800	0.064
B	1.000	0.952	0.922	0.958	0.531	0.873	0.831	0.643
C	0.840	0.997	0.491	0.840	0.875	0.482	1.000	0.289

&: a single chromosome out of 200 was found to carry SNP12 allele C; this chromosome was excluded from all LD analyses involving SNP12; $: for each LCR haplotype, ρ was calculated against the combination of the other two LCR haplotypes, thereby turning the LCR into a biallelic system.

Table 7. Results of EMSA assays that demonstrated allele-specific differential protein binding at the various SNP sites in the *GH1* gene promoter using rat pituitary cell nuclear extracts.

SNP	Position of double-stranded oligonucleotide	Sequence variation	No. of protein interacting bands			Transcription factor binding site/ functional region
			Strong	Medium	Weak	
8	-89 → -61	-75 A	-	1	-	Pit-1
		-75 G	1	1	-	Pit-1
9	-72 → -42	-57 T	1	-	-	Vitamin D receptor
		-57 G	2	-	-	Vitamin D receptor
10	-45 → -15	-31 G	1	-	-	TATA box
		-31 ΔG	-	-	1	TATA box
11,12,13	-18 → +15	-6/-1/+3 AAG	-	-	-	TSS
		-6/-1/+3 GAG	-	-	-	TSS
		-6/-1/+3 GTG	1	-	-	TSS
14,15	+4→+37	+16/+25 AA	2	1	-	5'UTR
		+16/+25 AC	2	-	-	5'UTR
		+16/+25 GC	1	-	-	5'UTR
		+16/+25 GA	2	1	-	5'UTR

TSS: Transcriptional start site 5'UTR: 5' untranslated region

Table 8 Association between adult height and *GH1* proximal promoter haplotype-associated *in vitro* expression data in 124 male Caucasians

	A_x<0.9	A_x>0.9
height<1.765	34	22
height>1.765	21	32

A_x: average normalized *in vitro* expression level of the two haplotypes of an individual i.e. $A_x=(\mu_{nor,h1}+\mu_{nor,h2})/2$.

Table 9 Average GC cell-derived, normalized luciferase activities ± standard deviation of different LCR-*GH1* proximal promoter constructs

Promoter haplotype	LCR haplotype			
	N	A	B	C
H1	1.00 ± 0.26^{x}	2.47 ± 0.41^{yz}	2.30 ± 0.46^{y}	2.77 ± 0.55^{z}
H23	1.00 ± 0.14^{x}	1.72 ± 0.55^{yz}	2.14 ± 0.52^{z}	1.35 ± 0.48^{xy}
H27	1.00 ± 0.26^{x}	1.11 ± 0.36^{x}	1.00 ± 0.41^{x}	1.25 ± 0.27^{x}

x,y,z: Tukey's studentized range test within a promoter haplotype; LCR haplotypes (A, B and C) with overlapping sets of letters are not statistically different in terms of their mean expression level. N: Construct containing proximal promoter but lacking LCR. LCR haplotypes were normalised with respect to N in each case.

Table 10 Two-way ANOVA of normalized luciferase activities of LCR-*GH1* proximal promoter constructs

Source	DF	Mean Square	F Value	Pr > F
Promoter haplotype	2	51.46	390.97	<.0001
LCR haplotype	3	5.67	43.08	<.0001
Interaction	6	3.09	23.48	<.0001

FIGURE LEGENDS

Figure 1: *GH1* gene promoter expression of negative controls as measured on different plates (a), and normalized expression levels of the wild-type haplotype (1), displayed as multiples of the plate-wise mean expression level of the wild-type (b).

Figure 2. Location of 16 SNPs in the *GH1* promoter relative to the transcriptional start site (denoted by an arrow). The hatched box represents exon 1. The positions of the binding sites for transcription factors, nuclear factor 1 (NF1), Pit-1 and vitamin D receptor (VDRE), the TATA box and the translational initiation codon (ATG) are also shown.

Figure 3: Normalized expression levels of the 40 *GH1* haplotypes relative to the wild-type (haplotype 1). Haplotypes associated with a significantly reduced level of luciferase reporter gene expression (by comparison with haplotype 1) are denoted by hatched bars. Haplotypes associated with a significantly increased level of luciferase reporter gene expression (by comparison with haplotype 1) are denoted by solid bars. Haplotypes are arranged in decreasing order of prevalence.

Figure 4: Minimum relative residual deviance $\delta_R(\Pi_{k,min})$ of normalized expression levels associated with haplotype partitioning using k SNPs (shaded bars). The dotted curve depicts the number of haplotypes comprising the minimum-δ_R-partitioning $\Pi_{k,min}$.

Figure 5: Relationship between size and cross-validated δ_R value for minimum deviance intermediate trees, using six selected SNPs (nos. 1, 6, 7, 9, 11 and 14). The dotted (horizontal) line corresponds to one SE of the cross-validated δ_R of the fully grown tree; the dashed

(vertical) line indicates the smallest tree for which the cross-validated δ_R lies within one SE of that of the fully grown tree.

Figure 6: Regression tree of *GH1* gene promoter expression as obtained by recursive binary haplotype partitioning, using six selected SNPs (nos. 1, 6, 7, 9, 11 and 14). Numbers on nodes refer to the SNPs by which the respective nodes were split. Terminal nodes ('leaves') are depicted as squares and numbered from left to right.

Figure 7: 'Reduced Median Network' connecting the seven haplotypes (circles) that have been observed at least 8 times in 154 male Caucasians. The size of each circle is proportional to the frequency of the respective haplotype in the control sample. Haplotypes H12 and H23 have been included as connecting nodes even although they have been observed only 5 and 2 times, respectively. SNPs at which haplotypes differ are given alongside each branch. The dark dot marks a non-observed haplotype or a double mutation at SNP sites 4 and 5.

Figure 8: Differences in protein binding capacity between *GH1* promoter SNP alleles revealed by electrophoretic mobility shift (EMSA) assays. Arrows denote allele-specific interacting proteins. The arrowhead denotes the position of a Pit-1-like binding protein. –ve (negative control), +ve (positive control), S (specific competitor), N (non-specific competitor), P (Pit-1 consensus sequence), P* (prolactin gene Pit-1 binding site), TSS (transcriptional initiation site).

Statement of the Invention

We describe herein a method of haplotype partitioning to identify mutations and/or polymorphisms that are major determinants of phenotype, particularly, but not exclusively, phenotype that is either advantageous or disadvantageous. For example, perhaps most typically, the method will be used to identify mutations and/or polymorphisms that are responsible, wholly or in part, for a physiological condition or disorder, such as, for example, a disease or abnormal or undesirable state.

Accordingly, the method of haplotype partitioning of the invention comprises examining the residual deviance (δ) for each mutation and/or polymorphism of a gene under consideration.

More ideally the method comprises examining the residual deviance (δ) of possible subsets of mutations and/or polymorphisms and so, most advantageously, the method is undertaken to examine the residual deviance (δ), upon haplotype partitioning {1...m}, of each possible subset of mutations and/or polymorphisms.

Most ideally still the method involves using the following function

$$\delta = \delta(\Pi) = \sum_{i=1}^{m} (\chi_i - \bar{\chi}_{\pi(i)})^2$$

(See page 11 for definitions)

The method of the invention is thought to be particularly, but not exclusively, suited to situations where the effects of said mutations and/or polymorphisms are strongly interdependent such as, for example, in the instance where there is linkage disequilibrium.

Using this methodology it is possible to identify those mutations and/or polymorphisms that are responsible for a sizeable proportion of the residual deviance in, for example, expression levels - where the mutations and/or polymorphisms are in the promoter region of the gene or, for example, protein function - where the mutations and/or polymorphisms are in the protein coding sequence of the gene.

Advantageously the methodology of the invention can be used to predict, and so subsequently make, super-maximal and sub-minimal haplotypes which may be useful, for example, as experiment controls in subsequent testing programmes.

Other methods for the identification of mutations and/or polymorphisms responsible for a sizeable proportion of the phenotype under consideration are described herein and constitute various aspects and/or embodiments of the invention.

According to a further aspect of the invention there is described herein significant mutations and/or polymorphisms, in the form of single nucleotide polymorphisms (SNPs), that are major determinants of the phenotype height.

More specifically, these SNPs, which are located in the proximal promoter of the growth hormone gene *(GH1)*, determine the level of expression of growth hormone and so the likely height of an individual.

It follows that knowledge of these SNPs or this subset of SNPs has utility in diagnostic techniques.

According to a further aspect of the invention there is provided a detection method for detecting a variation in *GH1* effective to act as an indicator of growth

hormone dysfunction in an individual, which detection method comprises the steps of:

(a) obtaining a test sample of genetic material from an individual to be tested, said material comprising, at least, a human *GH1* gene or a fragment thereof; and

(b) determining, and then, analysing the nucleotide sequence of said *GH1* gene, or fragment thereof, to see if any single nucleotide polymorphisms exist at any one or more of the SNP sites within the following SNP subset;

SNPs Nos. 1, 6, 7, 9, 11 and 14 (as described in Figure 2).

Alternatively, the above method may simply comprise determining whether there is a single nucleotide polymorphism at SNP7.

Ideally the diagnostic method of the invention comprises determining the nucleotide sequence of the *GH1* gene, or part thereof, in said test sample by sequencing methods employing PCR amplification of the *GH1* gene, or fragment thereof, using a nucleotide fragment that is specific for said *GH1* gene, or a part thereof.

Most ideally the diagnostic method of the invention involves PCR amplification of the proximal promoter region of the *GH1* gene and subsequent analysis of the amplified material to determine whether a single nucleotide polymorphism exist at one or more of the SNP sites designated 1, 6, 7, 9, 11 and 14.

In the instance where SNPs are identified at the aforementioned sites the diagnostic method is concluded by comparing the SNPs with published

information or that contained herein and determining whether a correlation means that there is a high likelihood that the individual being tested is under expressing growth hormone and is likely to suffer from known effects/complications associated with this physiology or over expressing growth hormone and so is likely to suffer from known effects/complications associated with this physiology.

As previously mentioned, using the haplotype partitioning method described herein it is possible to determine a super-maximal and sub-minimal haplotype and therefore the invention, according to a further aspect, also comprises the identification of a super-maximal and/or sub-minimal haplotype for the grown hormone gene.

The super-maximal haplotype (AGGGGTTAT-ATGGAG) is defined by a coding sequence that, in the embodiment described herein, enhances expression of growth hormone. Thus in the study herein described, the identification of proximal promoter SNPs in the grown hormone gene leads to increased promoter activity and so over expression of growth hormone. This in turn leads to an individual presenting a greater than average height. Conversely, the sub-minimal haplotype (AG-TTTTGGGGCCACT) defines a nucleotide sequence encoding a growth hormone gene whose promoter exhibits depressed activity and so overall production of growth hormone is reduced. An individual with this haplotype would be characterised by exhibiting a shorter than average height.

According to a further aspect of the invention there is therefore described a variant of *GH1* as described herein.

According to yet further aspects of the invention there is provided a screening method for screening an individual suspected of growth hormone dysfunction which screening method comprises the steps of :

(a) obtaining a test sample from an individual to be tested which sample contains genetic material comprising, at least, the growth hormone gene *GH1* or a fragment thereof;

(b) sequencing said gene, or fragment thereof;

(c) analysing said sequence for single nucleotide polymorphisms at any one of the following SNP sites within the proximal promoter (as illustrated in Figure 2 hereof) 1, 6, 7, 9, 11 and 14;

(d) comparing the identified single nucleotide polymorphisms with those in the published literature, or as described herein and determining whether a correlation means that said individual is likely to suffer from growth hormone dysfunction.

Alternatively, the above screening method may simply involve analysing said sequence for a single nucleotide polymorphism at SNP7.

Reference herein to growth hormone dysfunction includes reference to growth that is above or below average and requires knowledge thereof of treatment thereof by a clinician or patient.

According to further aspect of the invention there is provided a kit for carrying out the aforementioned diagnostic and/or screening methods. Ideally said kit contains the means for sequencing said growth hormone gene, or a part thereof and, optionally, information concerning said SNP sites 1, 6, 7, 9, 11 and 14 (as herein described).

The investigations related herein, centered on the growth hormone gene, led to the surprising conclusion that, in addition to the above identified major SNPs, there existed three additional SNPs upstream from the growth hormone gene, which had a significant role to play in determining growth hormone dysfunction and thus the likely height of an individual. These three SNPs were found to be within sites I and II of the upstream Locus Control Region (LCR) of *GH1*. These were located at nucleotide positions 990G, 1144A, 1194C. We ascribed to these three additional SNPs three distinct haplotypes and we have discovered that these haplotypes enhance proximal promoter activity by up to 2.8 fold depending upon the SNPs that exist in the proximal promoter, or put another way, depending on the haplotype of the proximal promoter.

Accordingly therefore, the aforementioned methods of the invention may, additionally or alternatively, comprise:

(a) obtaining a sample from an individual to be tested which sample comprises the genetic material encoding the Locus Control Region, or a part thereof, of the *GH1* gene.

(b) sequencing said Locus Control Region, or part thereof, in order to determine the genetic code thereof;

(c) analysing said sequence to determine whether it contains any one or more of the three SNPs located within sites I and II (990G, 1144A, 1194C) of the LCR region as described herein; and

(d) where said SNPs are present concluding that the LCR region of the growth hormone gene is likely to enhance proximal promoter activity and so increase expression of growth hormone.

Alternatively, said method may comprise simply analysing said sequence to determine whether a single nucleotide polymorphism exists at SNP7.

It will be apparent to one skilled in the art that once one has concluded that growth hormone is over expressed an individual is likely to grow to have, or have, above average height.

In an alternative aspect or embodiment of the invention the aforementioned methods may comprise, under step (d), concluding that where an individual has single nucleotide polymorphisms in any of the major determinant SNP sites in the proximal promoter and single nucleotide polymorphisms in said LCR region then said individual is likely to over express growth hormone and so grow to be, or be, above average height.

According to a further aspect of the invention there is provided the use of a growth hormone variant, as herein described, and in particular the use of a haplotypes of the proximal promoter region of the growth hormone gene, in the manufacture of a composition to treat growth hormone dysfunction. Further, there is provided a composition containing said variant or haplotype.

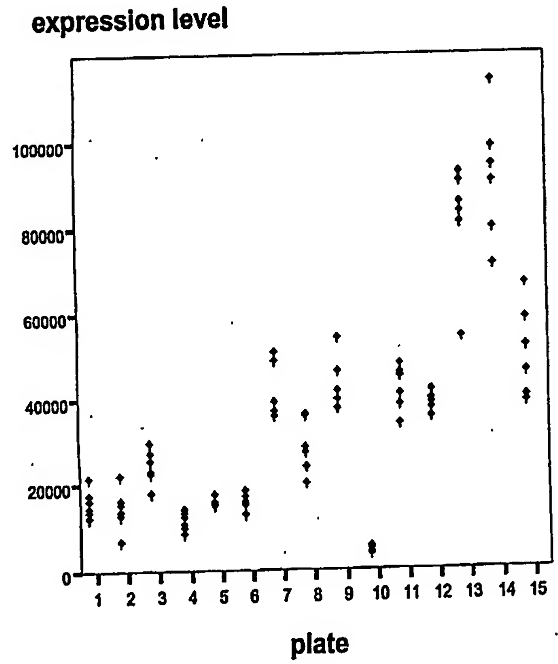
According to a further aspect of the invention there is provided the use of a LCR variant, as herein described, and in particular the use of a LCR haplotype in the manufacture of a composition to treat growth hormone dysfunction. Further there is provided a composition containing said LCR variant haplotype.

According to yet a further aspect of the invention there is provided a combination of a growth variant and a LCR variant, as herein described, in the manufacture of a composition to treat growth hormone dysfunction. Further, there is provided a composition containing said growth hormone variant and said

LCR variant.

Moreover, there is provided use of one or more of the subset of the proximal promoter haplotypes of *GH1* (SNPs 1, 6, 7, 9, 11 and 14) or the LCR haplotypes of *GH1* to treat growth hormone dysfunction.

(a)


expression level
100000
80000
60000
40000
20000
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
plate

(b)

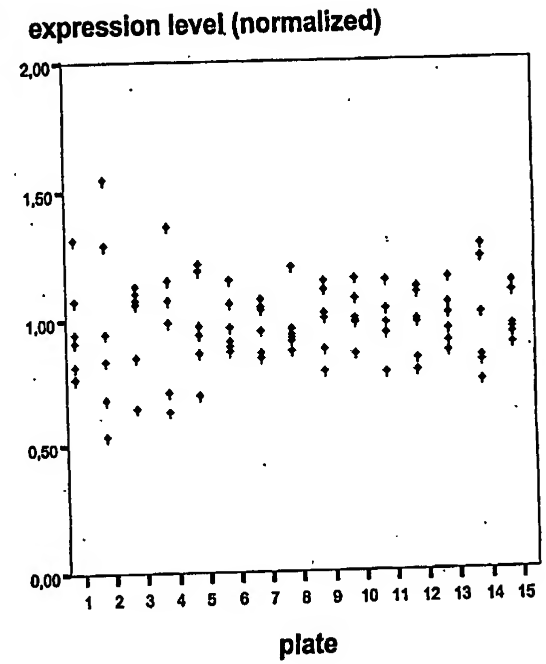

expression level (normalized)
2,00
1,50
1,00
0,50
0,00
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
plate

Figure 1.

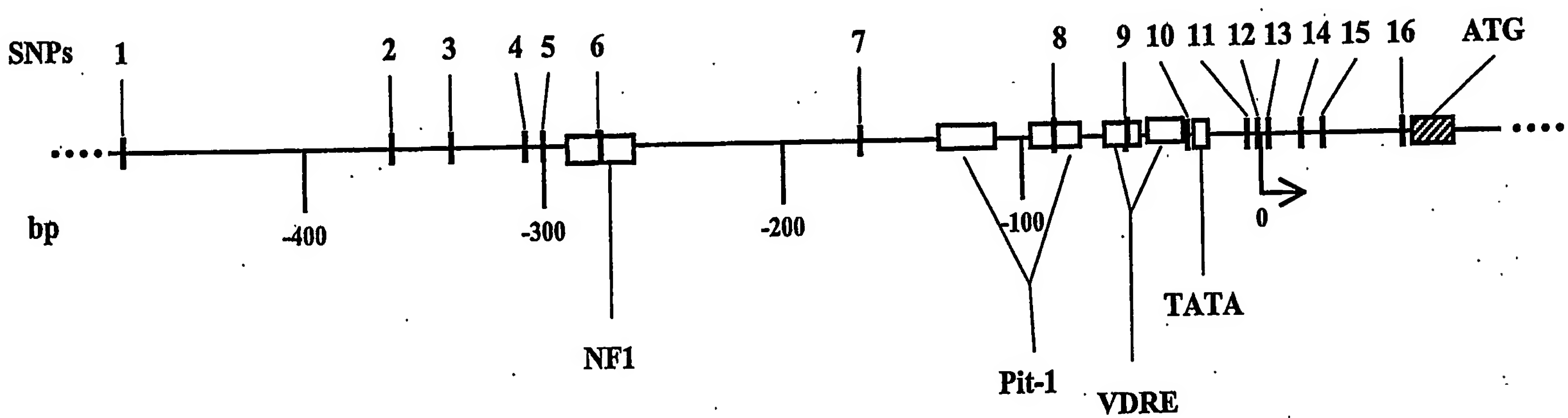

SNPs
1
2
3
4 5
6
7
8
9 10 11 12 13 14 15 16
ATG
bp
-400
-300
-200
-100
0
NF1
Pit-1
VDRE
TATA

Figure 2.

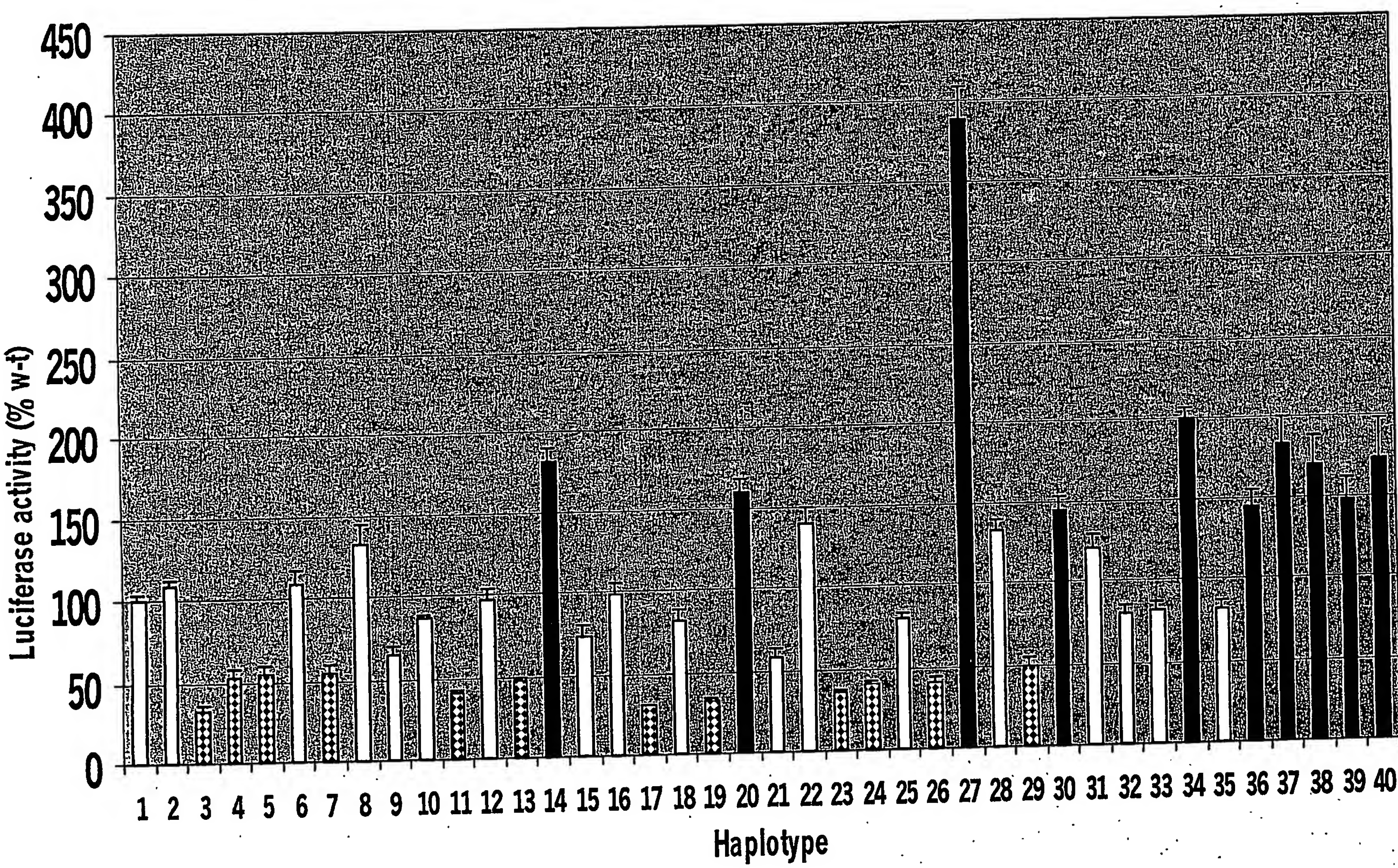

450
400
350
300
250
200
150
100
50
0
Luciferase activity (% w-t)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40
Haplotype

Figure 3.

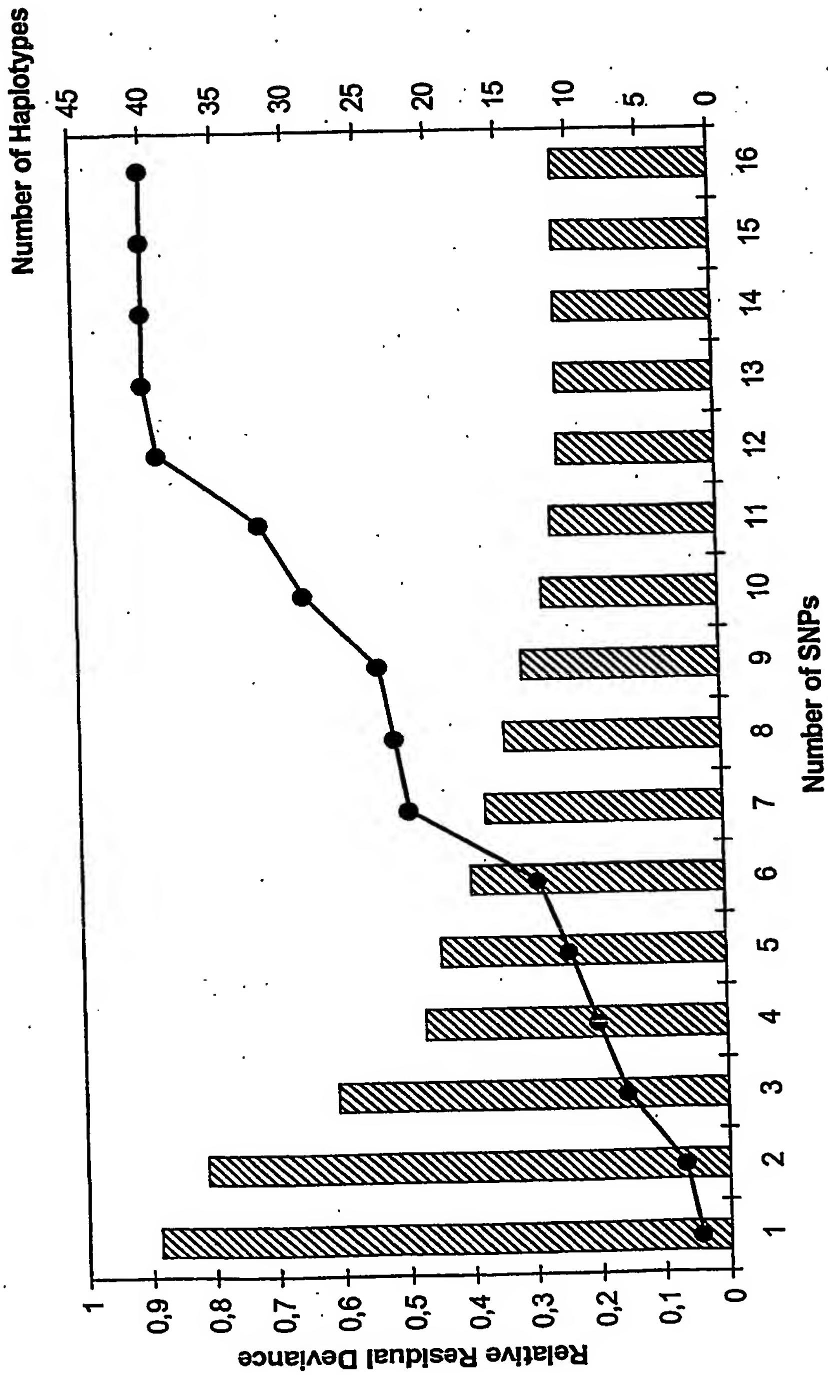

Number of Haplotypes
45
40
35
30
25
20
15
10
5
0
Relative Residual Deviance
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
Number of SNPs
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

Figure 4.

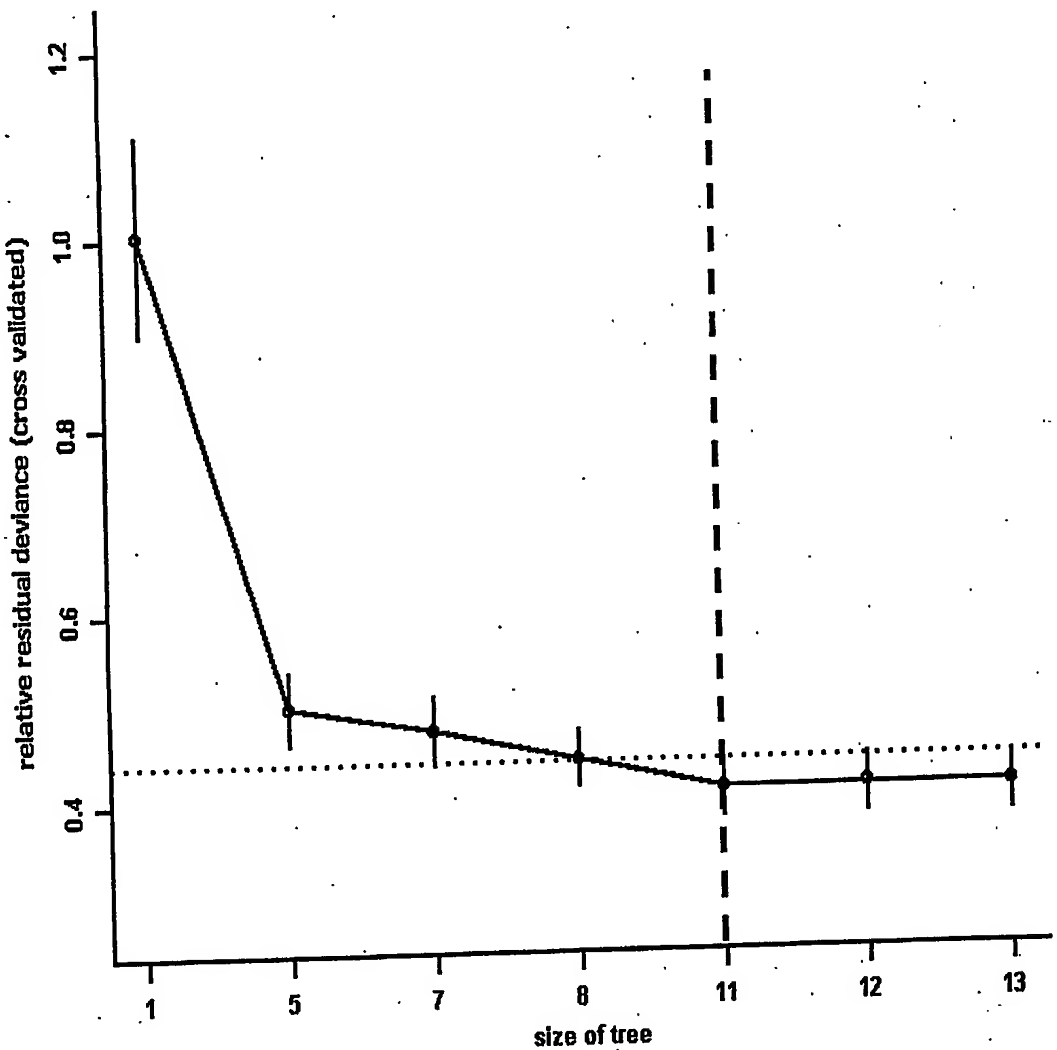

1.2
1.0
0.8
0.6
0.4
relative residual deviance (cross validated)
1
5
7
8
11
12
13
size of tree

Figure 5.

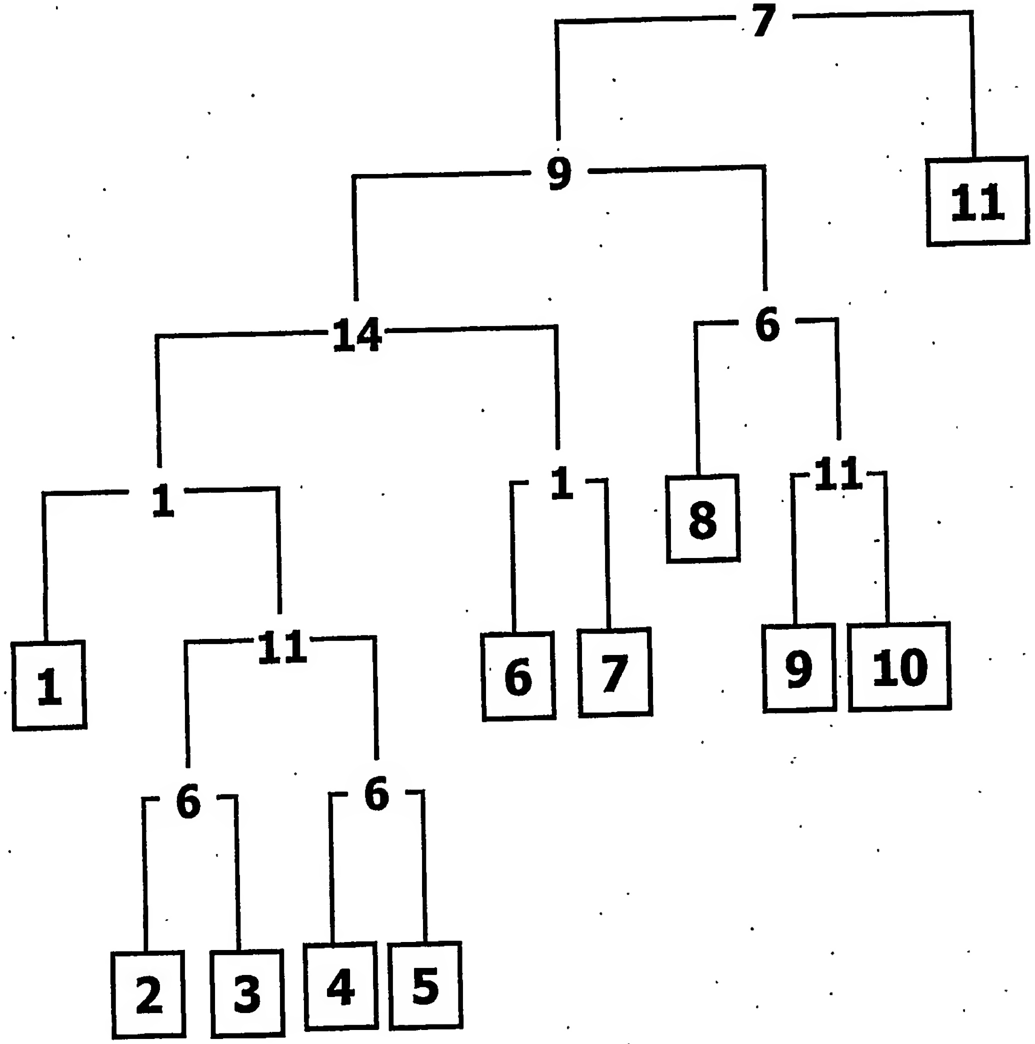


Figure 6.

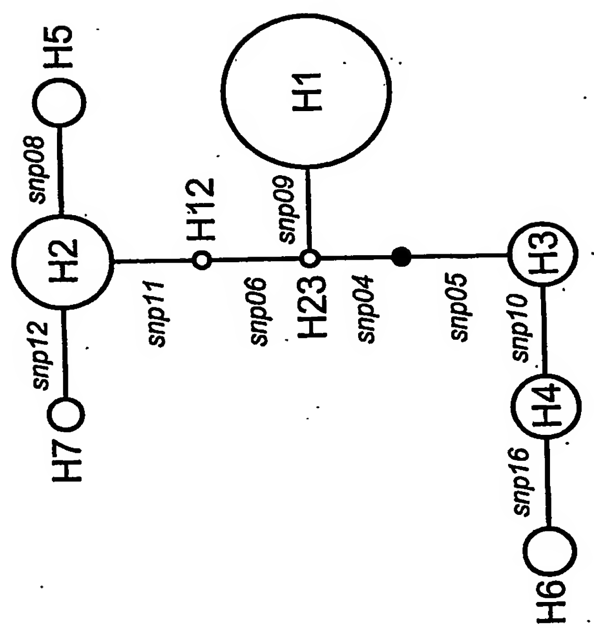

H5
H1
snp08
snp09
H12
H2
H3
snp11
snp06
H23
snp04
snp05
snp10
snp12
H4
H7
snp16
H6

Figure 7.

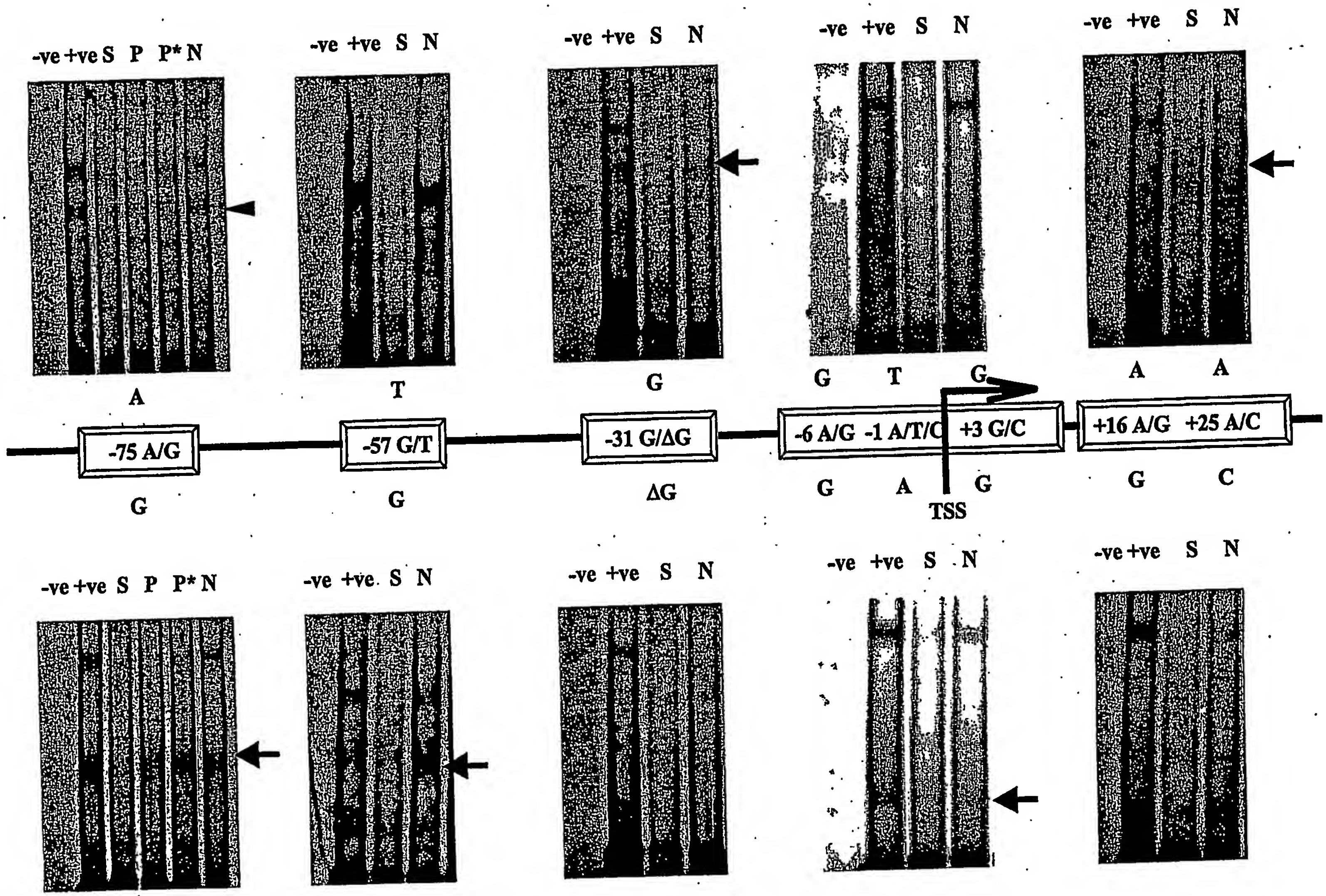

-ve +ve S P P* N
-ve +ve S N
-ve +ve S N
-ve +ve S N
-ve +ve S N
A
T
G
G T G
A A
-75 A/G
-57 G/T
-31 G/ΔG
-6 A/G -1 A/T/C +3 G/C
+16 A/G +25 A/C
G
G
ΔG
G A G
TSS
G C
-ve +ve S P P* N
-ve +ve S N
-ve +ve S N
-ve +ve S N
-ve +ve S N

Figure 8.